

The role of echolocation in communication in a high duty cycle echolocating bat, *Rhinolophus clivosus* (Chiroptera: Rhinolophidae): An experimental approach.

By Robert Raw



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I dedicate this thesis to my loving wife,

*Alicia Thomas-Rau*



# Table of Contents

Declaration	iii
Figure Index	iv
Abstract	viii
Acknowledgements	x
Chapter 1	1
Chapter 2	16
2.1 Study Area	16
2.2 Study Animals	17
2.3 Animal Capture and Husbandry	22
2.4 Preparation of Playback Calls	24
2.5 Habituation – Dishabituation Playback Experiments	25
Chapter 3	33
3.1 Introduction	33
3.2 Methods for Species Discrimination Trials	36
3.3 Results for Species Discrimination Trials	40
3.4 Discussion – Species Discrimination by <i>R. clivosus</i> using echolocation	48
Chapter 4	53
4.1 Introduction	53
4.2 Methods for Call Component Trials	55
4.3 Results for Call Component Trials	58
4.4 Discussion – Bats use multiple components of a signal for discrimination	66
Chapter 5	72
5.1 Introduction	72
5.2 Methods for Preference Trials	77
5.3 Results for Preference Trials.	84
5.4 Discussion – Preference for gender and body condition	92
Chapter 6	101
Chapter 7	107

## Declaration

I, Robert Nicholas Vause Raw, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I authorise the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever. This thesis has been submitted to the TurnItIn module and I confirm that my supervisors have seen my report and any concerns revealed by such have been resolved with my supervisors.

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# Figure Index

Figure 2.1 Cross Section of De Hoop Guano Cave (Adapted from McDonald et al. (1990b)). Numbers 1-7 represent site of temperature measurement (Laycock, 1983). Red triangles represent roost locations for Rhinolophids observed by McDonald et al. (1990b). Blue triangle represents primary location of *R. clivosus* capture for this study. .... 17

Figure 2.2 Geoffroy’s Horseshoe Bat, *Rhinolophus clivosus* ..... 18

Figure 2.3 Map of southern Africa showing the distribution of the three horseshoe bat species used in this study: *R. clivosus* (Rcl, dotted line, light grey; (Monadjem et al., 2010)), *R. blasii* (Rbl, dashed line, dark grey; (Monadjem et al., 2010)) and *R. capensis* (Rca, solid line, white; (Odendaal et al., 2014)). The study site, De Hoop Nature Reserve, is indicated by a red dot. Numbers indicate the resting frequencies of the three species. .... 19

Figure 2.4 Blasius’s Horseshoe Bat, *Rhinolophus blasii* ..... 20

Figure 2.5 Cape Horseshoe bat, *Rhinolophus capensis*..... 21

Figure 2.6 Layout of the bat housing room at the animal housing facility at UCT. .... 23

Figure 2.7 Experimental setup in the sound attenuating box (wooden crate, lined on all internal surfaces with sound absorbing foam. Internal dimensions: 77.5 x 38.0 x 38.0 cm), fitted with an ultrasound microphone and an ultrasound speaker, connected to a laptop. The box is monitored by an infrared video camera connected to an external monitor. A small perch is provided on the opposing wall to the speaker. .... 27

Figure 3.1 Discrimination of echolocation calls by *Rhinolophus clivosus*. Black indicates the percentage of trials in which the test subjects discriminated between habituation and test playbacks. Grey indicates trials in which test subjects generalised habituation and test playbacks as belonging to the same class of stimuli. Lines with asterisks indicate significant outcomes from post hoc comparisons (Fisher’s exact test, \*: P<0.05, \*\*: P< 0.005, \*\*\*: P<0.0005). The number of successful trials for each combination is displayed above the bars..... 42

Figure 3.2 Durations of attentive behaviours during the three analysed experimental phases (HabSTART (light grey), HabEND (no bar) and TEST (dark grey)), with boxes representing interquartile range and squares representing the median. Whiskers show non-outlier range within 1.5 times the interquartile range. Outliers outside of 1.5 times the interquartile range are presented with circles with extremes (over 2 times) represented by asterisks. The durations of attentive behaviours for the HabEND phase were close to zero and is just visible on this graph. Lines and asterisks indicate outcomes from all possible post hoc comparisons between the TEST phases (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ ). ..... 43

Figure 3.3 Plot of the first two functions of a DFA on ten acoustic parameters of playback stimuli. Each call of each playback class is plotted (N = 926) and each playback class is colour coded..... 47

Figure 4.1 Discrimination by *Rhinolophus clivosus* individuals between playbacks of individually controlled echolocation components. Black indicates the percentage of trials in which the test subjects discriminated between habituation and test playbacks. Grey indicates the percentage of trials in which test subjects generalised habituation and test playbacks as belonging to the same class of stimuli. Lines and asterisks indicate outcomes from all possible post hoc comparisons (Fisher’s exact test, \*:  $P < 0.0001$ ). The number of successful trials for each component is displayed above the bars..... 59

Figure 4.2 Durations of attentive behaviours during the three analysed phases (HabSTART (light grey), HabEND (no bar) and TEST (dark grey)), with boxes representing interquartile range. Whiskers show non-outlier range within 1.5 times the interquartile range. Outliers outside of 1.5 times the interquartile range are presented with circles with Extremes (over 2 times) represented by asterisks. The durations of attentive behaviours for the HabEND phase were close to zero and are barely visible on this graph. Lines and asterisks indicate statistical outcomes from all possible post hoc comparisons (\*:  $P < 0.01$ ). ..... 61

Figure 4.3 Plot of the first two functions of a DFA on ten acoustic parameters of the male and female playback stimuli. Each call is plotted (N = 1192) and colour coded for gender. .... 65

Figure 4.4 Plot of the first two functions of a DFA on ten acoustic parameters of the high bci and low bci playback stimuli. Each call is plotted (N = 1192) and colour coded for BCI..... 65

Figure 5.1 Experimental setup in the sound attenuating chamber viewed from the top (wooden box, lined on external surfaces with sound absorbing foam (solid lines) or plastic mesh (dashed line)). Internal dimensions: 125 x 125 x 125 cm. Top-Down view), fitted with an two ultrasound speakers, one on either side of a dividing wall broadcasting the playback stimuli simultaneously. The box was monitored by an infrared video camera placed approximately 2 m from the open end and connected to an external monitor. A small holding cage with trap door was positioned on the opposing wall to the speakers, from which the bat was released. .... 80

Figure 5.2 End zone sections set across the open end of the experimental box, viewed from the front. Each side of the dividing wall was subdivided down the middle to create an upper and lower half. The upper half was further subdivided to form a speaker section (a 10 cm perimeter immediately surrounding each speaker) and an upper section (The area above the middle line, excluding the speaker section). Each lower, upper and speaker sections comprised of 25%, 20% and 5% of the total end zone area respectively. .... 82

Figure 5.3 Percentage of landings for each stimulus by *Rhinolophus clivosus* males. Black indicates the percentage of landings at stimulus 1. Grey indicates the percentage of landings at stimulus 2... 88

Figure 5.4 Percentage of landings on either side of the box by *Rhinolophus clivosus* females. Black indicates the percentage of landings at stimulus 1. Grey indicates the percentage of landings at stimulus 2. .... 88

Figure 5.5 Durations spent by male *R. clivosus* at each stimulus, with boxes representing interquartile range and lines representing the median. Whiskers show non-outlier range within 1.5 times the interquartile range. Outliers outside of 1.5 times the interquartile range are presented with circles. .... 89

Figure 5.6 Durations spent by female *R. clivosus* at each stimulus, with boxes representing interquartile range and lines representing the median. Whiskers show non-outlier range within 1.5 times the

interquartile range. Outliers outside of 1.5 times the interquartile range are presented with circles.

..... 90

Figure 5.7 Relationship between BCI and eight different components of echolocation. No significant correlation was observed for any of the components ( $R^2$  and p values displayed on each graph).

..... 91

## Abstract

Acoustic communication plays a pivotal role in species recognition across a number of taxa. Species must therefore maintain discrete acoustic signatures to facilitate communication and avoid misidentification. The Acoustic Communication Hypothesis (ACH) thus proposes that in multispecies assemblages, multidimensional acoustic space is partitioned so that each species occupies a discrete acoustic space despite overlap in single parameters (e.g. frequency). Horseshoe bats use echolocation for the purpose of orientation and foraging. However, given the presence of individual and species specific cues in echolocation, it is likely that echolocation also functions to some degree in acoustic communication. This dual function makes echolocation a good model system to investigate the evolution of communication. In support of the ACH recent studies have shown that horseshoe bats are able to discriminate between conspecifics and heterospecifics based on echolocation calls alone even when call frequencies overlap. This suggests that multiple components of echolocation are influential in a bats ability to discriminate between species and indicates a dual function of echolocation in orientation and communication. These multiple components have not until now been identified. Studies have also suggested that preference for echolocation calls in the context of mate choice may influence the evolution of echolocation through sexual selection.

Using classical habituation – dishabituation playback experiments, I tested the ability of Geoffroy's horseshoe bat, *Rhinolophus clivosus*, to discriminate between echolocation calls of heterospecifics with either discrete or overlapping resting frequencies. I subsequently used synthesised calls in which I manipulated individual acoustic parameters, to investigate which call components are involved in discrimination amongst species. Finally, I used a two-

alternative choice experiment to investigate preference by *R. clivosus* to calls of individuals of opposite gender as well as between individuals with high or low body condition during the mating season.

*Rhinolophus clivosus* was able to discriminate readily between echolocation calls with discrete frequencies from different species. When frequencies overlapped, however, the ability of bats to discriminate between species was dependant on additional spectral and temporal components of calls that defined the entire acoustic space occupied by the calls. Consequently, calls that were similar in this multi-parametric acoustic space yielded low levels of discrimination, whereas calls separated in acoustic space yielded high levels of discrimination. This study provides the first experimental evidence of call components, other than frequency, that may play a role in species discrimination and questions earlier reports that bats use echolocation in mate choice. In support of the communicative role of echolocation, bats were also able to discriminate between calls from bats that differed in their body condition, indicating echolocation might represent an honest signal of mate quality and thus a function in mate choice. However, despite this, *R. clivosus* showed no preference or association based on gender or body condition. The results of this study suggest that despite having the ability to use echolocation to discriminate amongst conspecifics, these bats do not base mate choice on echolocation call components.

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# Chapter 1

## General Introduction and Literature Review

Communication can be defined as the provision of a signal or a cue from one organism, the sender, which stimulates the sensory systems of another organism, the perceiver, in a manner that provides information to the perceiver, resulting in a change in the behaviour of the perceiver (Ruxton and Schaefer, 2011). Animals use a wide range of signals to communicate, including visual, olfactory, tactile and acoustic cues. Such signals can serve a range of functions, including species recognition, conflict resolution, territory defence, sexual interactions, parent offspring interactions, social integration, and in auto-communication (Bradbury and Vehrencamp, 2010). The use of sound as a communication signal is common in a wide variety of animals but particularly prevalent in insects, frogs, birds, and mammals (Tyack, 1998; Gerhardt and Huber, 2002; Catchpole and Slater, 2008; Bradbury and Vehrencamp, 2010; Campbell et al., 2010; Konerding et al., 2011; Price, 2013; Kong et al., 2015). Acoustic signal design is influenced by all three components of a communication system; the producer of the signal, the environment, and the ability of the receiver to sense and interpret the signal (Bradbury and Vehrencamp, 2010).

As for all communication signals, acoustic signals play an important role in species discrimination and thereby in the life history of animals. In particular, a reliable identification of potential mates is vital for successful reproduction, especially in the presence of similar congeneric heterospecifics (Paterson, 1985), resulting in strong selection pressure for species to be able to distinguish conspecifics from sympatric similar species (Schuchmann and Siemers, 2010a). Failure to successfully discriminate between conspecifics and

heterospecifics may lead to a number of evolutionary costs, including wasted time in finding a potential mate and in the case of copulation occurring, unfit hybrids may be produced (Seddon and Tobias, 2010). Acting as an isolating mechanism between morphologically similar species, species recognition helps prevent excessive outbreeding which could result in the disruption of adaptive complexes (Jones, 1997; Braune et al., 2008b). As such, species recognition is thought to have evolved primarily to function in mate recognition and territorial defence (Bradbury and Vehrencamp, 2010). Most signals must therefore be species specific and distinctive enough to be reliably interpreted by the perceiver. Consequently, communication signals have been implicated in animal speciation in which communication signals function as ethological barriers which reproductively isolate one species from another. This has been reported for birds (Slabbekoorn and Smith, 2002; Balakrishnan and Sorenson, 2006; Förschler and Kalko, 2007), fish (Yamazaki and Goto, 2000; Leal and Losos, 2010), frogs (Fang et al., 2012; Kaefer et al., 2012) and mammals (Braune et al., 2008a; Jones, 2008; Campbell et al., 2010; Feoktistova et al., 2011). Species discrimination is therefore an important role of communication and has shaped the evolution of many communication signals (Ryan and Rand, 1993).

Acoustic signals have almost exclusively evolved to function in communication. There are only a few exceptions in which an acoustic signal has a function other than inter-individual communication. Echolocation of bats is such an exception. Echolocation is a sophisticated acoustic system that is used by only a few animals including bats and dolphins (Griffin, 1944; Schuller and Moss, 2004) for orientation and foraging (Neuweiler, 1989). Echolocation involves the complex integration of morphological and neurophysiological adaptations for the production, reception and interpretation of acoustic signals (Moss and Surlykke, 2010).

During orientation and foraging, an echolocating animal emits a series of sound pulses or calls which are reflected off objects as echoes. The special requirements of echolocation for the auditory pathway lie in the differences between the emitted call and the returning echo, which provide the animal with detailed information about its environment (Griffin, 1944; Fenton, 1984). Most echolocation calls of bats are in the range of ultrasound (Arch and Narins, 2008), because to detect small objects such as insects, the wavelength of the sound must be short which translates into high frequency. As with all acoustic signals, echolocation is heavily influenced by environmental factors because sound propagation is largely dependent on the characteristics of the medium through which it is transmitted and the structure of the surrounding habitat (Wiley, 1978). Therefore, the ultrasound echolocation calls of bats are very susceptible to environmental factors which influence sound attenuation due to increased scattering of higher frequency acoustic signals in the atmosphere (Wiley, 1978; Lawrence and Simmons, 1982). Insectivorous bat echolocation signals have therefore been adapted to the specific habitat in which bats forage and to their mode of food acquisition (Neuweiler, 1989; Barclay, 1999). It is firmly established that the spectral (e.g. frequency with maximum energy, bandwidth) and temporal (e.g. call duration) characteristics of echolocation calls are adapted to the exploitation of particular ecological niches (Neuweiler, 1989; Schnitzler and Kalko, 2001; Schnitzler et al., 2003). Given that many bat species utilize similar habitats and foraging modes to exploit similar prey items, the acoustic structure of echolocation signals in different species are often convergent, shaped by the similarities in the mode of foraging and habitat between species regardless of evolutionary relatedness (Jones and Teeling, 2006; Jacobs et al., 2013). Rhinolophid bats for example, require a long call that maintains a very specific frequency in order to detect fluttering wings of prey (Schnitzler and Denzinger, 2011). Such specificity of the call required for the primary function

of foraging creates a very narrow window for variation to accommodate communication cues, with a limited set of parameters that constitutes an ecologically viable bat (Kingston et al., 2000).

Echolocation calls may, therefore, lack the unique species, group or individual cues that are characteristic of communication signals (see for example Barclay (1999)). This, however, does not appear to be the case, as the acoustic structure of echolocation calls has been found to encode intraspecific signals carrying information about individual identity (Masters et al., 1995; Fenton et al., 2004; Carter et al., 2008; Yovel et al., 2009; Finger, 2015) and gender (Masters et al., 1995; Kazial and Masters, 2004; Knörnschild et al., 2012; Schuchmann et al., 2012; Finger, 2015) and colony membership (Pearl and Fenton, 1996; Boughman and Wilkinson, 1998; Esser and Schubert, 1998; Voigt-Heucke et al., 2010) as well as interspecific signals, including species identity (Jones and Van Parijs, 1993; Jones, 1997; Kingston et al., 2001; Mayer and von Helversen, 2001; Schuchmann and Siemers, 2010a; Li et al., 2014; Bastian and Jacobs, 2015). Echolocation, evolved in the context of orientation and foraging, may therefore also function in communication (Barclay, 1982; Fenton, 1985; Jones, 2008; Jones and Siemers, 2010; Schuchmann and Siemers, 2010a; Dorado-Correa et al., 2013; Bastian and Jacobs, 2015). One must consider, however, that these studies span across a broad range of species and that not all species exhibit distinct cues for these life-history traits (see Siemers et al. (2005)). The extent to which echolocation functions in communication is a subject of great interest in the study of the evolution of echolocation, resulting in a growing number of studies investigating various aspects of the field. It is widely considered that the communicative function of echolocation calls is largely via information leakage or “eavesdropping” (Barclay, 1982; Fenton, 2003; Gillam, 2007; Uebernickel et al., 2013).

Eavesdropping is a passive form of communication, in which an individual receives a signal from another individual which was not intended for that receiver. A prime example of eavesdropping is the use of feeding buzzes (a series of calls emitted during the final seconds of an attack on airborne prey) by a listening bat as an indicator of the presence of potential prey (Gillam, 2007; Dechmann et al., 2009). The use of another bats echolocation calls to find potential food sources is, however, not the only potential communicative role of echolocation. These readily available signals may reveal additional information about the signaller which can be used by the perceiver.

Subsequent to the discovery of the above mentioned “self-reporting signatures” (Smith and Harper, 1995) in echolocation calls, a few experimental studies tested the ability of bats to perceive these signatures and to use the information contained in them. Much of the work into species discrimination has been done using horseshoe bats (Rhinolophidae), one of the oldest bat families (Monadjem et al., 2010). These high duty-cycle, constant frequency (HD-CF) echolocating bats use calls that are characterised by long, narrowband echolocation pulses, separated by relatively short inter-pulse intervals (Neuweiler, 1989). These calls are dominated by a long duration ‘constant frequency’ (CF) component, flanked on either side by short duration ‘frequency modulated’ (FM) components. Horseshoe bats hearing is finely tuned to a specific range of frequencies known as the acoustic fovea, evident by a disproportionate representation of neurons in their auditory system associated with that particular range of frequencies (Schuller and Pollak, 1979). The narrow range of the acoustic fovea varies both amongst individuals of the same species and amongst different species. While in flight, Doppler shift causes a shift in frequency in the returning echo of an echolocation call to a moving bat. Horseshoe bats therefore use Doppler shift compensation

(DSC), a process in which a moving bat adjusts the frequency of the emitted call so that the returning echo falls within the acoustic fovea of that individual. Without DSC, a bat would not be able to hear the echo of its own call. As a consequence of DSC, bats emit calls at a frequency which is not audible in the acoustic fovea, avoiding self-deafening by very intense calls (Fenton, 1999). Additionally, DSC results in a constant echo from the background against which acoustic glints are generated when an incoming call is reflected off the fluttering wings of insects (Schnitzler and Denzinger, 2011). Horseshoe bats are ambush hunters and echolocate readily while at rest. The frequency of these calls, referred to as the 'resting frequency' (RF) is not affected by DSC and can be used as a standard frequency for comparison amongst individuals, populations and species. Hand-held bats therefore produce calls at the RF of the individual, making them the ideal family to investigate acoustic communication through the use of playback experiments (see section 2.4.1). The requirement for horseshoe bats to emit echolocation calls at frequencies which allow for returning echoes to fall within the acoustic fovea of a species led to the assumption that the frequency of the dominant CF component was crucial carrier of species specific signatures (Heller and Von Helversen, 1989; Jacobs et al., 2007; Schuchmann and Siemers, 2010a).

In the context of species discrimination in multi-species assemblages, an acoustic communication signal would have to allow discrimination amongst several species which also use similar acoustic signals. Such discrimination could be facilitated by signal divergence through the partitioning of acoustic signal space that minimizes overlap in the acoustic spaces used by the species and aids the ability of each species to distinguish conspecifics from heterospecifics. Resource partitioning of acoustic space is often reflected by the use of discrete frequency bands by species within a community (Duellman and Pyles, 1983; Heller and Von Helversen, 1989; Jacobs et al., 2007). Duellman & Pyles (1983) found that within a

given multispecies community of anurans, the available acoustic space is partitioned on the basis of ecological assemblages in which a species occurs. Gerhardt (1994), in a review of frog and toad vocalisations, found that anurans in multispecies assemblages displayed greater selectivity for specific frequencies than those in areas of allopatry. Females which failed to discriminate against calls in the extremes of the frequency band for the species might mate with males of another species, with the potential of disrupting any existing adaptive complexes. Selective pressures against such disadvantageous matings may have resulted in the stabilisation of frequency preferences for discrete frequency bands, facilitating intraspecific mate choice and avoiding hybridisation.

If echolocation calls are used for communication in rhinolophids, any individual using calls that are very different from those of conspecifics would not be able to communicate effectively. Therefore, social information in echolocation calls would need to be encoded via small intraspecific differences in calls (Heller and Von Helversen, 1989; Guillen et al., 2000). This is of further importance when considering the restriction enforced by the foraging and orientation function of echolocation, as the variations have to fall within the restrictions of the ecological functions (Kingston et al., 2000). Although variation is low within single populations, many rhinolophid species display geographic variation in echolocation frequency between communities, including *R. cornutus pumilus* (Yoshino et al., 2008), *R. monoceros* (Chen et al., 2009), *R. pusillus* (Jiang et al., 2010), *R. darlingi* (Jacobs et al., 2013), *R. clivosus* (Jacobs et al., 2007; Benda et al., 2008; Benda et al., 2010), *R. capensis* (Odendaal and Jacobs, 2011) and *R. blasii* (David Jacobs, unpublished data). Such geographic variation may arise from divergent character displacement to facilitate communication in different signalling communities. Character displacement of acoustic calls to avoid overlapping signals and

maintain discrete frequency bands in a new community may therefore allow for the geographic expansion of a species (Jones and Barlow, 2003). Character displacement likely facilitates intraspecific communication and may be responsible for divergence of call frequencies of rhinolophid (Russo et al., 2007) and hipposiderid bats (Thabah et al., 2006).

Evidence for possible character displacement in echolocation driven by communication can be found in the deviation of some species from an allometric relationship between body size and echolocation call frequencies in rhinolophids. Several families of both low duty cycle and high duty cycle echolocating bats, including the Rhinolophidae, display negative correlations between body size and peak echolocation frequency (Heller and Von Helversen, 1989; Barclay and Brigham, 1991; Jones, 1996). This is due to larger species having longer cords, which in turn results in the production of lower frequency signals (Fitch, 2006). However, there are notable deviations of some species from the allometric relationship between call frequency and body size for their family. In Europe, *R. mehelyi* echolocates at 30 kHz higher than would be expected from its body size (Puechmaille et al., 2014) and in Southern Africa, *R. clivosus* echolocates at 26 kHz above what would be expected (Jacobs et al., 2007). Species that deviate from the pattern provide ideal models to investigate how the evolutionary radiation of bats is influenced by echolocation, morphology and ecology (Jacobs et al., 2007).

Jacobs et al. (2007) investigated a number of hypotheses to explain the deviation of *R. clivosus* from the allometric relationship between call frequency and body size. They proposed that the 'acoustic communication hypothesis' (ACH, Duellman and Pyles (1983)) offers the best explanation for the deviation from allometry observed in the species. The ACH (Heller and Von Helversen, 1989; Kingston et al., 2000; Thabah et al., 2006; Jacobs et al., 2007)

in its original form proposes that the relatively high or low echolocation frequencies used by some ecologically similar rhinolophid species evolved under selection pressure through competition for private frequency bands during social interactions, allowing for effective intraspecific communication and facilitating recognition of conspecifics. The establishment of *R. clivosus* in new communities in which there is competition between congeners for distinct frequency bands, may have therefore been possible due to character displacement, resulting in a shift in the frequency band used *R. clivosus* to one not used by others in the community (Jones and Barlow, 2003; Jacobs et al., 2007).

Similarly, geographic variation in call frequency in *R. blasii* may also be the result of the same process. In southern Africa, *R. blasii* emits echolocation calls at two different peak frequencies, corresponding with the presence or absence of *R. clivosus* in a given population. In areas where *R. blasii* occurs without *R. clivosus*, it emits echolocation calls of 90 kHz - 92 kHz (David Jacobs unpublished data, Rbl<sub>92</sub>), the same range of frequencies used by *R. clivosus* (90 kHz - 92 kHz, Jacobs et al. (2007), Rcl<sub>92</sub>). However, in areas where *R. blasii* co-occurs with *R. clivosus*, *R. blasii* is found to echolocate at frequencies of 85 kHz - 87 kHz (David Jacobs unpublished data, Rbl<sub>87</sub>). The lower frequencies used by *R. blasii* populations in sympatry with *R. clivosus* may therefore be the result of competition for private frequency bands, necessary to maintain discrete communication pathways between species.

The ACH, as originally outlined, relies on two primary assumptions: firstly, that echolocation has a communicative function and secondly, that frequency is the primary component of echolocation driving the communicative function. The importance of signal frequency as a communication cue has also been demonstrated in a number of animal groups, most notably anurans (Duellman and Pyles, 1983; Gerhardt, 1994).

Initial studies investigated interspecific discrimination in horseshoe bats and focused on the frequency of the CF component as the primary cue for discrimination. Schuchmann and Siemers (2010a) demonstrated that two species of horseshoe bats, *R. mehelyi* and *R. euryale*, were both able to discriminate calls of congeneric heterospecifics from those of conspecifics. Both species were also able to discriminate between calls of two congeneric heterospecifics. In both cases, the frequency of the CF component of the echolocation calls were focused on as the primary recognition cue, as the ability to discriminate was reduced when frequency bands overlapped completely. Still, the authors concluded that the ability of bats to discriminate overlapping calls, although with reduced capacity, suggests that other components of echolocation are influencing species recognition. Further evidence was provided by Li *et al.* (2014) for three Rhinolophid species, *R. macrotis*, *R. lepidus* and *R. sinicus*. Again, the frequency of the CF component was assumed to be the primary recognition cue. The importance of the call frequency was however questioned as no correlation was found between acoustic parameters of the calls and the behavioural response of the bats. The emphasis on frequency, therefore, may have been slightly over-estimated.

With growing evidence for the ability of horseshoe bats to discriminate between species despite similar call frequencies, it is clear that additional components of the calls are being used for discrimination. The focus of research has begun to shift towards investigating which components of echolocation could serve as cues for communication, over and above call frequency. Schuchmann and Siemers (2010a) initially suggested the terminal FM components of the calls to be a likely candidate. Bastian and Jacobs (2015) further demonstrated the ability of horseshoe bats to use multiple call components to discriminate between species using two phonetic populations of *R. capensis* and revealed potential acoustic parameters used by bats.

They demonstrated that certain geographic populations of *R. capensis* were able to discriminate calls from both allopatric and sympatric horseshoe bat species with discrete and overlapping frequencies to their own species. This finding supports the notion that horseshoe bats use multiple components of echolocation to discriminate between species when call frequencies are similar. The use of multiple components of an acoustic call for communication in bats is not a surprising notion, considering the extent of the variation observed in bat social calls, which are not constrained by an alternate function. Social calls have been observed to vary across a number of parameters to portray specific information, including frequency, duration, and call shape (Pfalzer and Kusch, 2003; Ma et al., 2006). Although echolocation does not have the potential to show the same level of variation as in social calls, it is still likely that the variation that is available is used by bats as communication cues.

It is clear that the original formulation of the ACH was deficient in its focus on call frequency as the determining factor for resource partitioning in horseshoe bat echolocation. Bastian and Jacobs (2015) proposed the following alternative formulation of the ACH to account for multiple call components in which they refer to acoustic space defined by multiple call components including frequency: "In an assemblage of sympatric heterospecific animals using acoustic signals, multidimensional acoustic space is partitioned in such a way that each species occupies a distinct acoustic space facilitating intraspecific communication and species discrimination". Although Schuchmann & Siemers (2010a) and Bastian & Jacobs (2015) reasoned which additional parameters of echolocation calls may serve as additional discrimination cues based on an acoustic analysis of calls, no study has directly experimentally tested which parameters of echolocation calls these may be. Through an analysis of the acoustic complexity of the different calls used in the study, Bastian and Jacobs (2015)

determined that call duration, duration to maximum intensity, minimum frequency of final FM component and call entropy are all potentially important components for communication but did not provide a test as to whether they are in fact perceived by bats.

Bastian and Jacobs (2015) also found that different geographic populations of the same species differed in their perceptual acuity and ability to detect small variations in acoustically similar calls. A population of the same species occupying a relatively simple acoustic assemblage (only one sympatric species with non-overlapping frequencies and very few populations of conspecifics with variable call frequencies) failed to discriminate between conspecifics and heterospecifics when frequencies overlapped. In contrast, populations from more complex acoustic assemblages (presence of sympatric heterospecific, *R. clivosus*, with partially overlapping call frequencies as well as multiple populations of conspecifics with variable call frequencies in close geographical proximity) could discriminate amongst sympatric heterospecific with overlapping call frequencies. They concluded that bat populations from more complex acoustic assemblages had an increased perceptual acuity to account for the complexity in the acoustic space around them. As a result, they were able to discriminate between calls of acoustically similar species by perceiving minute acoustic differences. In contrast, populations of the same species from regions of relatively simple acoustic assemblage had not evolved the same level of perceptual acuity and were therefore unable to discriminate between the same sets of acoustically similar calls.

The evolution of an acoustic signal such as echolocation into a communication trait, requires a benefit to the sender and in some degree to the receiver. In the absence of a benefit to survival, there would be no driving force in the co-option of echolocation signals for communication. The case for the ACH and echolocation as a means of communication can

be strengthened if the discrimination that echolocation allows is used by the bats to enhance their survival or reproductive success. Body condition index (BCI) can be used as an indicator of fitness and thus may play a role in mate choice and ultimately in reproductive success of an individual. Puechmaille *et al.* (2014) found a strong link between echolocation frequency and BCI in the horseshoe bat *R. mehelyi*. They then proceeded to experimentally demonstrate that females preferentially selected calls from males with a higher BCI, giving behavioural support for the role of echolocation in mate choice in this species.

In the context of bat communication via echolocation, the validity of the ACH is dependent on at least three assumptions, 1) the ability of bats to discriminate between species on the basis of echolocation alone, 2) on the use of echolocation cues other than frequency to facilitate such discrimination, and 3) the use of this discriminatory ability to enhance their survival or reproductive success. Although several studies (e.g. Schuchmann and Siemers (2010a), Li *et al.* (2014) and Bastian and Jacobs (2015)) have tested two of the three assumptions of the ACH separately on several different horseshoe bat species, no single study has tested all three assumptions on the same species. Testing individual assumptions on different species, forces one to extrapolate findings for one species across all species. This runs the risk of falsely accepting an assumption which may not be applicable to the species in question. Testing all three assumptions on the same species, however, eliminates this risk and allows for the development of a more reliable theory.

The geographic variation in echolocation of *R. blasii*, in relation to the presence or absence of *R. clivosus*, as described above, provides a useful system to further investigate the role of frequency in communication. If *R. clivosus* is able to discriminate calls of both phonetic populations of *R. blasii* from calls of conspecifics, it is likely that *R. clivosus* uses multiple

components of echolocation for species identification and not just frequency, which would support the revised formulation of the ACH (Bastian and Jacobs, 2015). Alternatively, if *R. clivosus* is able to discriminate the calls of Rbl<sub>87</sub>, but not those of Rbl<sub>92</sub>, it would suggest that without the separation in call frequency, there is insufficient variation between *R. clivosus* and *R. blasii* for discrimination, when both species occupy the same acoustic space.

In this study, multiple experimental approaches are used on *R. clivosus* to explore questions relating to the three assumptions of the ACH:

1. Assumption 1: Can bats discriminate between echolocation calls of different species despite frequency overlap?

H<sub>1</sub>: *R. clivosus* can discriminate the echolocation calls of congeneric heterospecific bats from those of both con- and heterospecifics, regardless of overlapping frequencies.

H<sub>0</sub>: *R. clivosus* does not discriminate between the echolocation calls of congeneric heterospecific bats from those of both con- and heterospecifics.

2. Assumption 2: If bats are able to discriminate between signals that overlap in call frequency, which acoustic components of the call in addition to frequency carry discrimination cues?

H<sub>1</sub>: *R. clivosus* can discriminate between echolocation calls based on individual components of echolocation besides frequency.

H<sub>0</sub>: *R. clivosus* does not discriminate between echolocation calls which overlap in frequency despite varying in other components.

3. Assumption 3: Are echolocation calls used as cues for reproductive fitness (e.g. BCI) and are such cues used to choose high quality mates?

H<sub>1</sub>: *R. clivosus* show preference for echolocation calls produced by individuals of the opposite gender with good body condition.

H<sub>0</sub>: *R. clivosus* shows no preference for echolocation calls produced by individuals with good body condition.

## Chapter 2

### General Methods

#### 2.1 Study Area

All test subjects were collected from the De Hoop Guano Cave within De Hoop Nature Reserve (34°26'S, 20°25'E, Figure 2.3) in the Western Cape of South Africa. Due to safety concerns and equipment requirements, cave exploration and animal capture was restricted to parts of the cave not used as a 'maternity chamber' (Figure 2.1) by female bats. The majority of *R. clivosus* were captured in a medium sized chamber just short of the maternity chamber. Experiments were conducted at two locations: species recognition trials were conducted at an animal housing facility in the Department of Biological Sciences of the University of Cape Town (UCT) and echolocation component discrimination and mate choice trials were conducted at the De Hoop field research station situated within De Hoop Nature Reserve, approximately 10km from De Hoop Guano Cave.

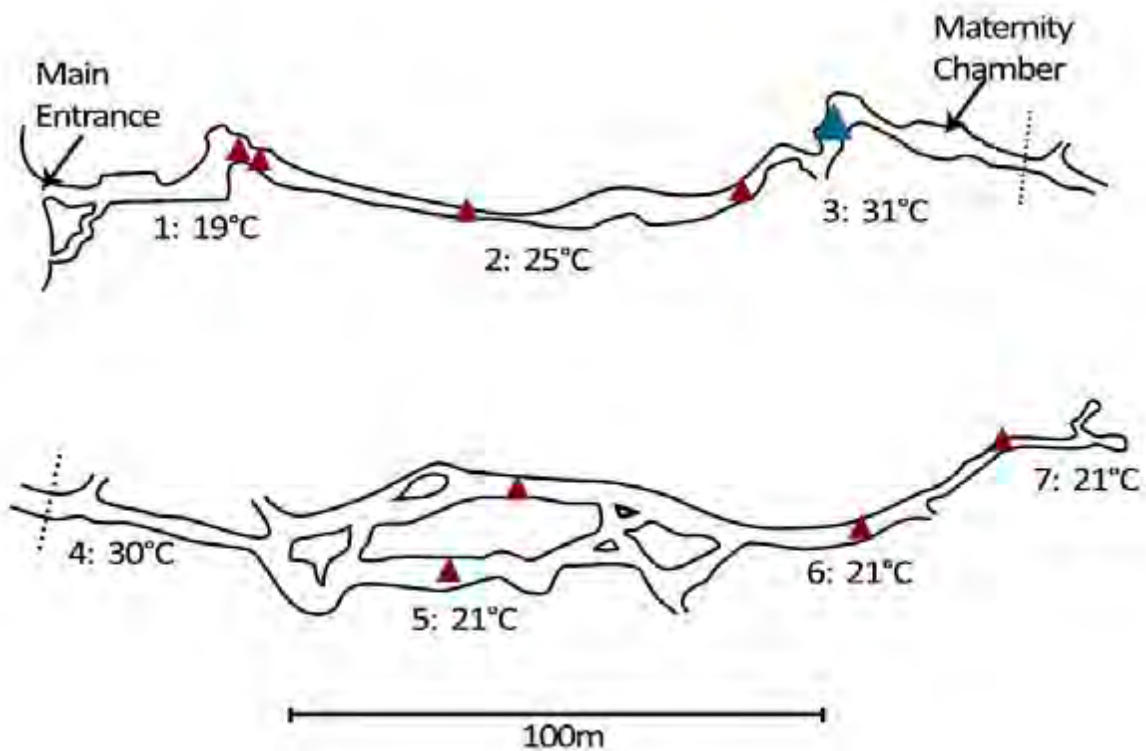


FIGURE 2.1 CROSS SECTION OF DE HOOP GUANO CAVE (ADAPTED FROM McDONALD ET AL. (1990B)). NUMBERS 1-7 REPRESENT SITE OF TEMPERATURE MEASUREMENT (LAYCOCK, 1983). RED TRIANGLES REPRESENT ROOST LOCATIONS FOR RHINOLOPHIDS OBSERVED BY McDONALD ET AL. (1990B). BLUE TRIANGLE REPRESENTS PRIMARY LOCATION OF *R. CLIVOSUS* CAPTURE FOR THIS STUDY.

## 2.2 Study Animals

High duty-cycle, constant frequency (HD-CF) echolocating bats, such as those of the family Rhinolophidae, are characterised by long, narrowband echolocation pulses, separated by relatively short inter-pulse intervals (Fenton, 1999). The narrow bandwidth of high duty cycle echolocation pulses allows for more distinct separation between frequency bands than would be visible in low duty cycle bats. They are also perch hunters and can reliably echolocate from a stationary position, making it easy to record their echolocation calls and to conduct playback experiments with them. In this study, I used *Rhinolophus clivosus* as a focal species to which I played back recorded echolocation calls from two other rhinolophids, *R.*

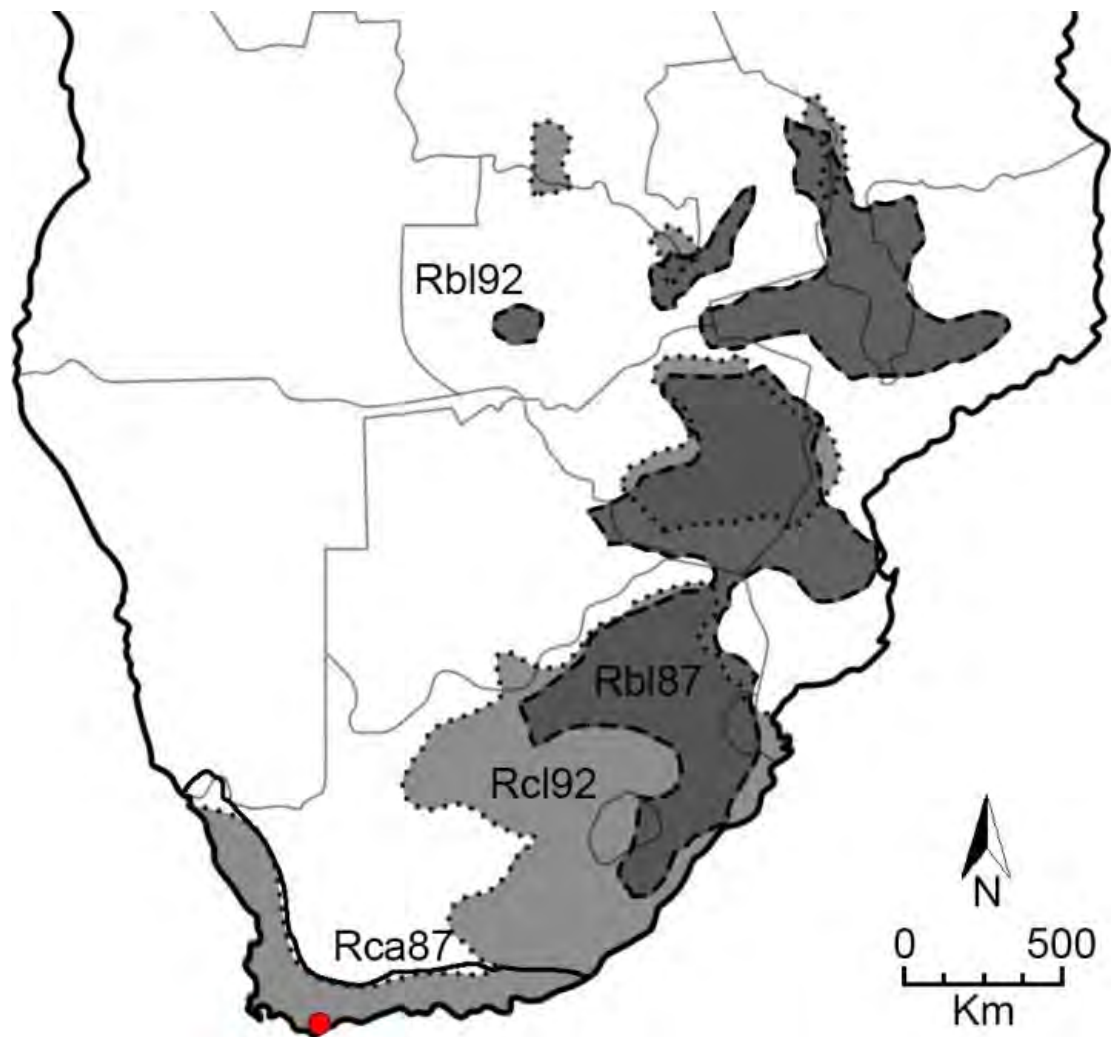
*blasii* and *R. capensis*, which are sympatric with *R. clivosus* over most, but different parts, of its geographic range.

### 2.2.1 *Rhinolophus clivosus*

*Rhinolophus clivosus* (Figure 2.2), is a medium sized insectivorous bat (forearm length (FA): 53 mm, (Schoeman and Jacobs, 2003; Jacobs et al., 2007; Monadjem et al., 2010)) with a wide distribution throughout the eastern parts of southern Africa and along the southwestern coastal belts (Figure 2.3) (Monadjem et al., 2010), extending up through Africa to the Mediterranean (Benda et al., 2010). It has much variation in echolocation frequencies across its geographical range. *Rhinolophus clivosus* in southern Africa produce HD-CF echolocation calls at a RF of 90 kHz to 92 kHz (Jacobs et al., 2007) (“Rcl<sub>92</sub>”), whereas towards the northern extent of its range, *R. clivosus* is observed to echolocate at frequencies 5-6 kHz lower (84-87 kHz) (Benda et al., 2008; Benda et al., 2010) than southern populations.



FIGURE 2.2 GEOFFROY'S HORSESHOE BAT, *RHINOLOPHUS CLIVOSUS*



**FIGURE 2.3** MAP OF SOUTHERN AFRICA SHOWING THE DISTRIBUTION OF THE THREE HORSESHOE BAT SPECIES USED IN THIS STUDY: *R. CLIVOSUS* (RCL, DOTTED LINE, LIGHT GREY; (MONADJEM ET AL., 2010)), *R. BLASII* (RBL, DASHED LINE, DARK GREY; (MONADJEM ET AL., 2010)) AND *R. CAPENSIS* (RCA, SOLID LINE, WHITE; (ODENDAAL ET AL., 2014)). THE STUDY SITE, DE HOOP NATURE RESERVE, IS INDICATED BY A RED DOT. NUMBERS INDICATE THE RESTING FREQUENCIES OF THE THREE SPECIES.

### 2.2.2 *Rhinolophus blasii*

*Rhinolophus blasii* (Figure 2.4) is a smaller rhinolophid (FA: 46mm, (Monadjem et al., 2010)) and is distributed widely across Africa and Europe. In southern Africa, *R. blasii* is sparsely distributed throughout the eastern regions (Figure 2.3), partly overlapping with the distribution of *R. clivosus*. As with *R. clivosus*, *R. blasii* emits HD-CF echolocation calls which show geographic variation in call frequency. In areas where *R. blasii* occurs in the absence of

*R. clivosus*, *R. blasii* uses a frequency band overlapping with that of *R. clivosus* (RF: 90 kHz - 92 kHz “Rbl<sub>92</sub>”; (this study)). However, in areas where both species co-occur, *R. blasii* uses a lower frequency band of 85 kHz - 87 kHz (“Rbl<sub>87</sub>” (Monadjem et al., 2007; Schoeman and Jacobs, 2008)).



FIGURE 2.4 BLASIUS’S HORSESHOE BAT, *RHINOLOPHUS BLASII*

### 2.2.3 *Rhinolophus capensis*

*Rhinolophus capensis* (Figure 2.5) is a medium sized rhinolophid (FA: 46-51.8 mm,(Jacobs et al., 2007)) endemic to the Cape Floristic Region in the south Western Cape of South Africa, extending as far as southern Namibia (Figure 2.3), often occurring in sympatry with *R. clivosus* but is never sympatric with *R. blasii* (Monadjem et al., 2010). The frequency used by *R. capensis* varies across its distribution range (RF: 87 kHz “Rca<sub>87</sub>”; (Odendaal et al., 2014;

Bastian and Jacobs, 2015)) thus it partly overlaps with that of Rbl<sub>87</sub>, but does not overlap with that of Rcl<sub>92</sub>. This natural system allows us to test whether *R. clivosus* can discriminate between two congeners (Rca<sub>87</sub> & Rbl<sub>87</sub>) which use calls of similar frequency but which do not overlap with its own. We also test whether *R. clivosus* can discriminate between two populations of the same congeneric species using different frequencies (Rbl<sub>87</sub> & Rbl<sub>92</sub>), one of which overlaps with its own.



**FIGURE 2.5 CAPE HORSESHOE BAT, *RHINOLOPHUS CAPENSIS*.**

## 2.3 Animal Capture and Husbandry

### 2.3.1 Animal Capture

Bats were captured using hand nets inside De Hoop Guano Cave. The capture and handling complied with the guidelines recommended by the American Society of Mammalogists (Sikes et al., 2011) and Kunz and Parsons (2009). Work was conducted under Cape Nature permit (AAA007-00113-0056) and University of Cape Town Animal ethics clearance (2013/2012/V33/DJ). Bats were individually marked with a colour coding system on their toe nails using non-toxic nail polish.

### 2.3.2 Captive Colony

Immediately after capture, adult *R. clivosus* were temporarily housed at the De Hoop research station for two nights in a well-ventilated, mesh tent with free access to water. During the first two nights after capture, the health of each bat was assessed at the field station and the strongest males were transported to the UCT animal housing facility, where they were housed for the duration of the study (Duration: five to six days). Bats were transported in individual cotton bags suspended within a wire mesh transportation cage. Only male bats were kept for the captive colony of *R. clivosus* to avoid removing pregnant or lactating females from the roost.

At the UCT animal housing facility, bats were housed in a large indoor enclosure (3.3 m x 3 m x 2 m), lined with 5 mm mesh polyethylene netting (Figure 2.6). Temperature within the room was controlled at  $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$ . Humidity was maintained between 50% and 80%. Day-night cycles were controlled artificially using red lights connected to an automated timer switch. Cycles were shifted by two hours per day resulting in a partially inverted day-night cycle. In captivity, all captured bats were fed daily with fortified mealworms and the health

and weight of each bat was assessed daily. After a period of captivity, bats were inspected by a registered veterinarian before being released at the site of capture.

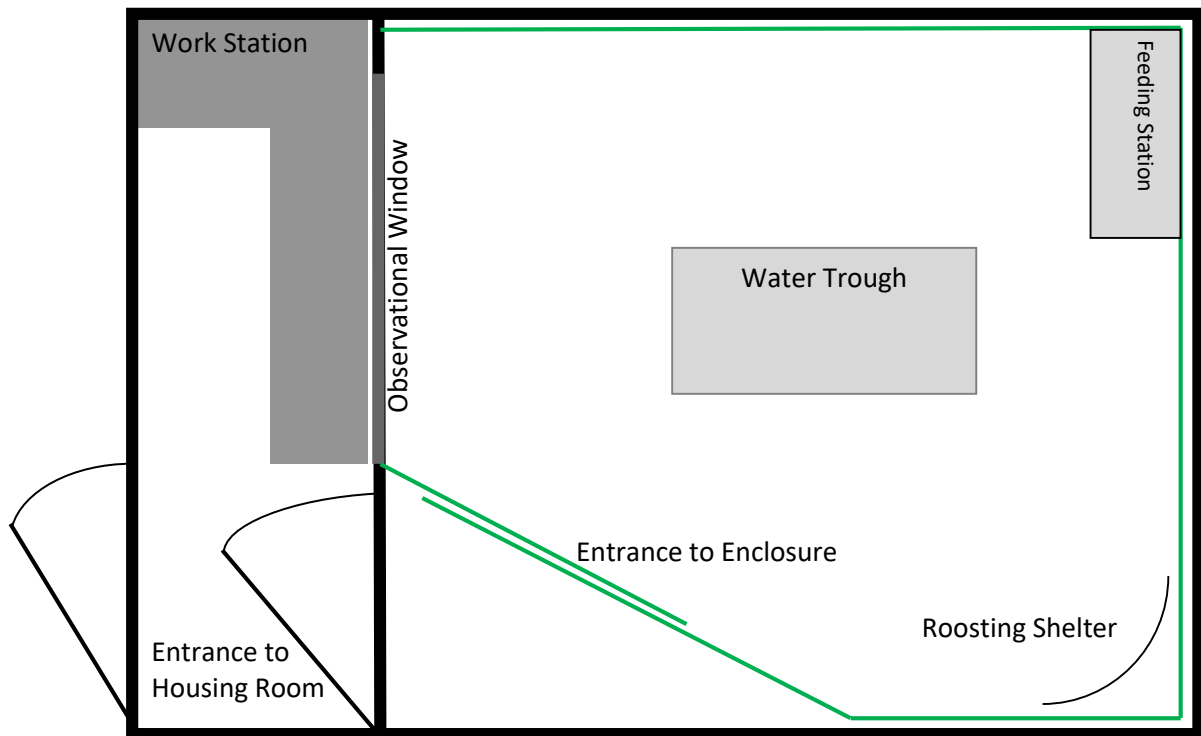


FIGURE 2.6 LAYOUT OF THE BAT HOUSING ROOM AT THE ANIMAL HOUSING FACILITY AT UCT.

### 2.3.3 Field Colony

For the experiments done at the De Hoop field station, both male and female adult bats were used because pups had by that stage been weaned and mating had yet to occur, so there was no danger of inadvertently keeping pregnant or lactating females in captivity. Bats were housed in an indoor flight room (3 m x 2.5 m x 2 m) with free access to water. Captive bats were again fed with fortified mealworms and the health and weight of each bat was assessed daily. Field colonies were kept for two to three nights before being released near the site of capture.

### 2.3.4 Short Term Experiments in the Field

Bats captured for mate choice experiments at the De Hoop field station were housed in a wire-mesh transportation cage, lined with soft mesh netting. Bats were provided free access to water and released at site of capture within 12 hours.

## 2.4 Preparation of Playback Calls

### 2.4.1 Playback Experiments

Playback is an experimental technique used to observe an animal's behavioural response to an acoustic signal in isolation of other stimuli. The technique involves the rebroadcasting of a signal, either natural or synthetic, to a listening animal and observing their behavioural response (McGregor, 1992). Playback studies allow for researchers to observe a response to a specific acoustic signal without the confounding effects of additional natural factors associated with the signaler (i.e. visual, tactile, olfactory cues) to determine the effect of the acoustic signal alone (Falls, 1992). Playback studies have been used on a variety of animal groups, including birds (Irwin and Price, 1999; Otter et al., 1999), small mammals (English et al., 2008; Kessler et al., 2012), marine mammals (Deecke, 2006), human infants (Flom and Pick, 2012) and, in line with the current study, bats (Schuchmann and Siemers, 2010a; Li et al., 2014; Bastian and Jacobs, 2015).

### 2.4.2 Echolocation recordings

Playback stimuli were prepared using an existing database of echolocation sequences which were recorded from captured hand-held bats using the Avisoft UltraSoundGate 416 (Avisoft Bioacoustics, Glienicke, Germany), with a sampling rate of 500 kHz or the Pettersson D1000X (Pettersson Elektronik, Uppsala, Sweden) with a sampling rate of 340 kHz.

Echolocation calls were selected based on the quality of the call, selecting for calls with good signal to noise ratio. A signal to noise ratio (SNR) was calculated for each call using the equation

$$SNR = 10\text{Log} \left( \frac{P_{signal,dB}}{P_{noise,dB}} \right)$$

where  $P_{signal,dB}$  is the power of the signal and  $P_{noise,dB}$  is the power of the noise measured for the same duration of the signal right before or after the call. A call was said to have a good signal to noise ratio if the SNR value was  $> 1$ . Any calls with a  $SNR < 1$  were discarded. The first five calls in a sequence were avoided because rhinolophid bats tune into their frequency after a period of silence (Schuller and Suga, 1976; Siemers et al., 2005).

### 2.4.3 Semi-Synthetic Call Duplication

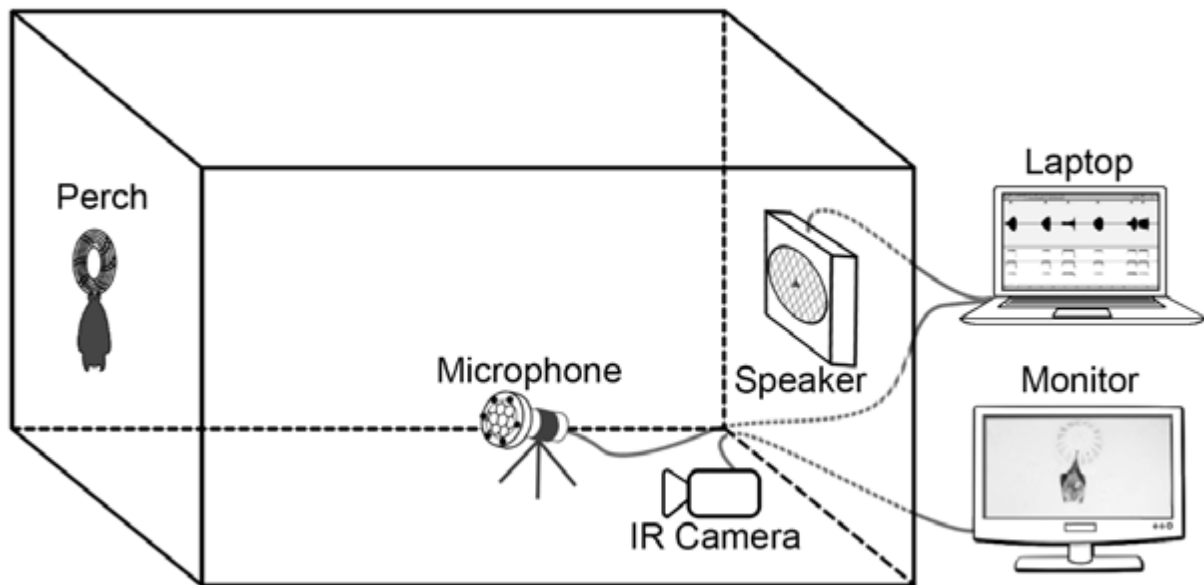
To reduce unwanted noise or recording artefacts influencing the bats response to the playbacks, semi-synthetic duplicates of all selected calls were produced following the parameters of the dominant second harmonic of the original call using Avisoft-SASLab Pro (v.5.2.07, Avisoft Bioacoustics, Glienicke, Germany). The fundamental and third harmonics were created artificially based on the relative amplitudes for these harmonics to the dominant harmonic. The synthesised calls were then compiled into a series of playback files for each experiment (outlined in detail in sections 3.2, 4.2 and 5.2).

## 2.5 Habituation – Dishabituation Playback Experiments

### 2.5.1 Experimental Procedures

Habituation-dishabituation experiments (Eimas et al., 1971) were conducted on adult *R. clivosus* individuals. Experimental trials took place in a sound attenuating box (Figure 2.7). The

setup was the same as in Bastian and Jacobs (2015) which is based on that in Schuchmann & Siemers (2010a). Playbacks were produced using an ultrasound speaker (Avisoft UltraSoundGate Player BL Light, Avisoft Bioacoustics, Glienicke, Germany). Behavioural responses were recorded using an infrared video camera (Bell + Howell DNV16HDZ, New York, USA), while acoustic responses were recorded using an ultrasound microphone (Avisoft UltraSoundGate 416, Avisoft Bioacoustics, Glienicke, Germany) and Avisoft-Recorder software (Version 4.2, Avisoft Bioacoustics, Glienicke, Germany) on a Lenovo Ideapad laptop (Model Y580). Video and audio recordings were synchronised using the terminal white noise control, visible on the audio line of both the video and audio software. Call intensities were normalised using the automated normalisation function (Avisoft-SASLab Pro, v.5.2.07, Avisoft Bioacoustics, Glienicke, Germany) within and across all playbacks. Output intensities of the playbacks were checked using recordings of the playbacks from within the experimental chamber and compared with natural calls. Output intensities were controlled using Avisoft Recorder (Avisoft Bioacoustics, Glienicke, Germany) and amplifier volume and gain were kept constant for all trials. Test playbacks were terminated with a 0.02 s broadband white noise as a control for false negative responses (i.e. bats having fallen asleep, experimental fatigue) (Pancratz and Cohen, 1970). A fade-in from 0 dB to maximum intensity was used across the duration of the first call in each playback file, to avoid crackling when the speaker starts at a high intensity.



**FIGURE 2.7 EXPERIMENTAL SETUP IN THE SOUND ATTENUATING BOX (WOODEN CRATE, LINED ON ALL INTERNAL SURFACES WITH SOUND ABSORBING FOAM. INTERNAL DIMENSIONS: 77.5 X 38.0 X 38.0 CM), FITTED WITH AN ULTRASOUND MICROPHONE AND AN ULTRASOUND SPEAKER, CONNECTED TO A LAPTOP. THE BOX IS MONITORED BY AN INFRARED VIDEO CAMERA CONNECTED TO AN EXTERNAL MONITOR. A SMALL PERCH IS PROVIDED ON THE OPPOSING WALL TO THE SPEAKER.**

Bats were placed on the perch and allowed time to habituate to the sound-attenuating box in silence. Habituation was defined as a point where a bat was silent and immobile at the perch for at least 20 s. Immobility includes no crawling activity, no ear movements, no head movements and no stretching of wings or legs. Habituation was determined visually and subjectively during the trials and confirmed statistically subsequently. Once habituated to the box, the habituation playback was presented to the bat, and the bat was allowed to habituate to this playback. When the bat showed no activity for 20 s, i.e. was habituated, the playback was changed to the test playback. There was a pause of approximately 150 ms during the playback changeover due to unavoidable delays in computer processing of the playback. The habituation file was placed on loop until the time of changeover in the event that the bat took longer than 300s to habituate. Trials were stopped if the bat was not habituated after 60 minutes.

Each experimental trial contained a single playback combination, consisting of a habituation and test signal. The order in which bats were tested was randomised daily (resulting in each bat being tested at a different time each night) as was the order of presentation of playback classes (resulting in each playback combination being presented at a different time slot each day) to avoid any effect of trial order on behavioural responses to any particular individual or playback category.

### 2.5.2 Behavioural Responses: Video Analysis

The bat's behavioural response was measured from video recordings for 20 s at three time frames across each trial: onset of habituation (HabSTART), final period of habituation before playback was changed (HabEND) and onset of the test playback (TEST). To control for false negative discrimination responses (e.g. experimental fatigue), only trials in which the bat reacted to both the onset of habituation and the terminal white noise were considered to be successful and therefore included in the analyses.

The Solomon Coder (Version 14.05.18, András Péter, Hungary, [www.solomoncoder.com](http://www.solomoncoder.com)) was used to record the duration and frequency of 17 behaviours (Table 2.1) that were observed during video analysis of captive *R. capensis* held at the animal housing facility at UCT. Behaviours were categorised as either attentive or inattentive. Attentive behaviours were defined as behaviours associated with listening, searching or echolocation as well as behaviours observed in direct response to external stimuli (e.g. sudden loud noise or experimental stimuli). Inattentive behaviours (e.g. stretching, grooming, etc.) were defined as behaviours that were not performed in response to an external or experimental stimuli or not linked to any behaviour regarded as attentive. Statistical analysis focused on the eight attentive behaviours: Rapid Ear Twitching (RET), Slow Ear Twitching (SET), Full Head Lift (FHL),

Partial Head Lift (PHL), Partial Leg Contractions (PLC), Full Leg Contractions (FLC), Scan (SC) and Echolocation Calls (EC). For the statistical analyses the durations of all above listed attentive behaviours were combined (referred to as “Attentive”). As single behaviours sometimes overlapped in time (i.e. head movements and ear twitching occurring simultaneously), I avoided simply adding the durations, instead I discarded overlaps and coded a single attentive variable incorporating any attentive behaviours. The strength of a behavioural response was defined using duration of the combined attentive behaviours.

Bats were regarded as having discriminated between the habituation and test playbacks if any attentive behaviour was observed in the first 5 s of the TEST. If no attentive behaviours were observed, bats were regarded as having generalised the habituation and test playbacks as belonging to the same class.

**TABLE 2.1 LIST OF BEHAVIOURS CODED FROM VIDEO AND/OR AUDIO RECORDS DURING HABITUATION-DISHABITUATION EXPERIMENTS**

<b>Behaviour</b>	<b>Class</b>	<b>Description</b>	<b>Type</b>
Rapid Ear Twitching (RET)	Attentive	Continuous rapid movement of entire ear(s) towards sound source, single twitches cannot be counted in real time.	Duration
Slow Ear Twitching (SET)	Attentive	Continuous movement of entire ear(s) towards sound source, single twitches can be counted in real time.	Duration
Full Head Lift (FHL)	Attentive	Complete lifting of the head, to a point where the chin is visible, with no sideways movement or twitching of the ears.	Duration
Partial Head Lift (PHL)	Attentive	Partial raising of the head, to a point where the chin still cannot be seen.	Duration
Scan (SC)	Attentive	Continuous rapid movement of entire ear(s) towards the sound source and head fully lifted looking left and right.	Duration
Full Leg Contraction (FLC)	Attentive	Legs completely contracted by bending at the knee, often associated with hunching of body.	Duration
Partial Leg Contraction (PLC)	Attentive	Legs partially contracted by bending at the knee, often associated with hunching of body.	Duration
Echolocation Calls (EC)	Attentive	Count of echolocation pulses.	Count
Single Ear Twitch (SET)	Inattentive	Single movement of the ear(s).	Count
Tip of Ear Twitch	Inattentive	Single movement of only the tip of the ear.	Count
Head Swaying	Inattentive	Side to side movement of the head, without the head being lifted.	Duration
Body Shuffle	Inattentive	Side to side movement of the body without relocation of feet, usually ending in sleeping position.	Duration
Crawling	Inattentive	Repositioning along the perch, involving movement of feet and wings, settling in another position.	Duration
Vibrating Wings	Inattentive	Rapid vibrations of both wings, usually ending in a sleeping position.	Duration
Full Wing Stretch	Inattentive	Full length expansion of wing(s) to the side.	Count
Partial Wing Stretch	Inattentive	Partial expansion of wing(s) to the side.	Count
Grooming	Inattentive	Use of tongue or feet to groom fur and wings.	Duration

### 2.5.3 Statistical Analysis

All statistical testing was conducted using Statistica 64 (Version 11, StatSoft Inc., Tulsa, USA) with a significance level of  $\alpha=0.05$ . Shapiro-Wilks tests were used to test for normality in the data to determine if non-parametric tests should be used. The Shapiro-Wilks test was used because of its efficiency with smaller sample sizes. A Friedman ANOVA was used to analyse the potential long-term effects of the experimental repeats over multiple nights on the duration of attentive behaviours. The durations of attentive behaviours during HabSTART across all trials was used.

To test if bats were indeed fully habituated to the habituation stimulus before playing back the TEST stimulus, the duration of attentive behaviours during HabSTART and HabEND were compared for each playback combination using Wilcoxon Pairs Tests. To test if bats discriminated between the habituation playback and the test playback, the duration of attentive behaviours during HabEND and TEST were compared for each playback combination using Wilcoxon Pairs Tests.

To test if the strength of behavioural response differed between playback combinations, the number of trials in which the bats discriminated the test playback from the habituation playback was compared between all playback combinations using a Pearson Chi Squared Test. Posthoc pairwise comparisons were conducted using Fishers Exact test to examine the number of successful discriminations between all permutations of the five playback combinations. Bonferroni corrections were not applied during post hoc comparisons due to the impact of such manipulations on statistical power, particularly of analyses involving small sample sizes (Nakagawa, 2004).

To test if there was a difference in the strength of response between playback combinations, the duration of attentive behaviours in response to the test playback was compared using a Kruskal-Wallis ANOVA, with all post hoc multiple pairwise comparisons made between each playback combination. Individual attentive behaviours were then further analysed to determine if the strength of any particular behaviours differed between trials using Kruskal-Wallis ANOVA, with post hoc comparisons compiled for all significantly differing behaviours.

Inter- and intra-observer reliability tests were used to test for potential observer bias in video analysis. Ten videos were selected at random and re-coded by an external observer or for the intra-observer reliability test by the same observer, and the results were compared with those of the original coding using Kappa Coefficients for frequency data and Wilcoxon Pairs Test for duration. Both inter- and intra-observer biases were discounted and a high agreement confirmed (Kappa Coefficient: Inter:  $K = 0.87$ ,  $N = 10$ ,  $P > 0.05$ ; Intra:  $K = 0.85$ ,  $p > 0.05$ . Wilcoxon Pairs Test: Inter:  $Z = 0.62$ ,  $p > 0.05$ ; Intra:  $Z = 0.64$ ,  $p > 0.05$ ).

# Chapter 3

## Species Discrimination

### 3.1 Introduction

Species recognition is an essential function of communication, necessary for an individual to identify conspecifics for intraspecific social interaction and cooperation not relating to reproduction that can lead to fitness benefits for an animal (Alcock, 2001). To recognise species, an individual first needs to be able to discriminate at least between signals of conspecifics and heterospecifics, and then must match signals to particular species. Discrimination of conspecifics from heterospecifics alone does not, therefore, necessarily imply recognition of particular species. Species recognition is also important in the identification of an appropriate mate (Ryan and Rand, 1993). Mate choice is considered to be a primary driving force behind species recognition (Bradbury and Vehrencamp, 2010). An inability to identify appropriate mates may be costly to an individual, potentially leading to failed copulation attempts or the production of unfit hybrids (Seddon and Tobias, 2010). This is particularly important in the presence of similar congeneric heterospecifics (Paterson, 1985).

Acoustic signals are central to communication systems of many animal groups, including insects (Claridge and de Vrijer, 1994; Gerhardt and Huber, 2002), fish (Danley et al., 2012), anurans (Gerhardt, 1994; McLean et al., 2013), birds (Slabbekoorn and Smith, 2002; Catchpole and Slater, 2008; Price, 2013) and mammals (Campbell et al., 2010; Konerding et al., 2011; Teichroeb et al., 2013). Much of the focus on acoustic communication has been on anurans and birds. The majority of species specific signals emitted by anurans are advertisement calls

by males which serve both to attract females and to acquire or defend territories from other males (Gerhardt, 1994). These calls contain species specific cues and can act as isolating mechanisms among sympatric species (Duellman and Pyles, 1983). Likewise, in bird communities, birdsong can act as an important isolating mechanism (Slabbekoorn and Smith, 2002). Species specific songs are often used during courtship displays to attract females, resulting in song playing a prominent role in mate selection (Slabbekoorn and Smith, 2002; Riebel, 2009; Helfer and Osiejuk, 2015).

Bats emit two fairly distinct forms of acoustic signals: social calls and echolocation. Echolocation functions primarily in orientation and foraging and is adapted for the exploitation of particular ecological niches (Neuweiler, 1989; Schnitzler and Kalko, 2001; Schnitzler et al., 2003) due to the influence of environmental (Wiley, 1978) and ecological factors on call design. This results in convergent evolution of echolocation calls in ecologically similar species (Jacobs et al., 2013). Despite the convergence between similar species, echolocation calls have been shown to act as cue for species identity across a number of bat species (Jones and Van Parijs, 1993; Jones, 1997; Kingston et al., 2001; Mayer and von Helversen, 2001; Schuchmann and Siemers, 2010a; Li et al., 2014). Echolocation may, therefore, also function in communication (Barclay, 1982; Fenton, 1985; Jones, 2008; Jones and Siemers, 2010; Schuchmann and Siemers, 2010a; Dorado-Correa et al., 2013; Bastian and Jacobs, 2015). A communicative function of echolocation may play an important role in identifying conspecifics for the location and membership of social aggregations (Kunz, 1982).

Much of the work on species discrimination by echolocation has been done using horseshoe bats. The utilisation of distinct frequency bands by horseshoe bats in multispecies assemblages may be a result of selection to facilitate intraspecific communication. This is

similar to the situation observed in anurans, where call frequency is considered as the most important communication cue (Duellman and Pyles, 1983; Gerhardt, 1994). This has led to the formulation of the acoustic communication hypothesis which proposes that partitioning of acoustic signal space to minimize overlap may aid in the ability of each species to distinguish conspecifics from heterospecifics (Heller and Von Helversen, 1989; Kingston et al., 2000; Thabah et al., 2006; Jacobs et al., 2007; Bastian and Jacobs, 2015). In the context of bat communication, the validity of the ACH is dependent on at least three assumptions, 1) the ability of bats to discriminate between species on the basis of echolocation alone, 2) on the use of echolocation cues other than frequency to facilitate such discrimination, and 3) the use of this discriminatory ability to enhance their survival or reproductive success.

Interspecific discrimination has been demonstrated in a number of horseshoe bat species, including *Rhinolophus macrotis*, *R. lepidus* and *R. sinicus* (Li et al., 2014), *R. mehelyi*, *R. euryale* (Schuchmann and Siemers, 2010a) and *R. capensis* (Bastian and Jacobs, 2015). These studies, however, all demonstrated that the use of discrete frequency bands among co-existing species was not entirely necessary for successful discrimination, although the strength of discrimination was reduced when frequencies overlapped completely (Schuchmann and Siemers, 2010a; Li et al., 2014). The ability to discriminate when calls partially overlap suggests that multiple components of echolocation are being used for discrimination (Schuchmann and Siemers, 2010a; Bastian and Jacobs, 2015). Bastian and Jacobs (2015) further extends this to propose that bats from more complex acoustic assemblages have an increased perceptual acuity, allowing for discrimination between acoustically similar echolocation calls that would otherwise be indiscernible to bats from relatively simple acoustic assemblages.

In this chapter I investigate whether *R. clivosus* can discriminate echolocation calls of both conspecifics and heterospecifics with both overlapping and discrete frequency bands, testing the first assumption of the ACH. Echolocation calls from two phonic groups of *R. blasii* (one overlapping in resting frequency (92 kHz) with those of the listening *R. clivosus* and one discrete (87 kHz)) were used together with calls of the congeneric heterospecific *R. capensis*, echolocating at a discrete frequency from *R. clivosus*, but overlapping with the lower frequency phonic group of *R. blasii* (87 kHz).

H<sub>1</sub>: *R. clivosus* can discriminate the echolocation calls of congeneric heterospecific bats from those of both con- and heterospecifics, regardless of overlapping frequencies.

H<sub>0</sub>: *R. clivosus* does not discriminate between the echolocation calls of congeneric heterospecific bats from those of both con- and heterospecifics.

## 3.2 Methods for Species Discrimination Trials

### 3.2.1 Preparation of Playback Files

Recordings from *R. blasii* were split into two categories, based on different populations of this species using discrete ranges of resting peak echolocation frequencies from 86 kHz - 88 kHz (Rbl<sub>87</sub> – Gatkop Cave, South Africa) from 91 kHz - 93 kHz (Rbl<sub>92</sub> – Kalenda, Zambia). A single category from a single population of each of the other two species *R. clivosus* (Rcl<sub>92</sub> – De Hoop Nature Reserve) and *R. capensis* (Rca<sub>87</sub> – Table Farm, South Africa) was also created, resulting in a total of four playback stimuli categories (Rcl<sub>92</sub>, Rbl<sub>92</sub>, Rbl<sub>87</sub>, Rca<sub>87</sub>), based on species and resting echolocation frequency (RF).

A set of 200 echolocation calls (n) from different individuals (N) were selected from each species (Rcl<sub>92</sub>, N=20, n=10; Rbl<sub>92</sub>, N=6, n=34; Rbl<sub>87</sub>, N=10, n=20, Rca<sub>87</sub>, N=20, n=10). To avoid

pseudoreplication, equal numbers of calls from both male and female individuals were used in the playback recordings to preclude test subjects discriminating between playbacks on the basis of gender instead of species. Similarly, the use of calls from multiple individuals precluded individual-based discrimination and ensured any discrimination was species discrimination.

These semi-synthetic calls were then compiled in random order (calls from all individuals combined) to minimise the effect of individual recognition cues. Two playback files, a habituation (duration = 300 s) and a test (duration = 20 s) playback file, were created for each of the four categories. Individual inter-pulse intervals (IPI) separating the calls were based on the IPI preceding the call in the original recorded sequence.

The four different playback classes were grouped into five playback combinations in which the first class represents the habituation class and the second class represented the test class i.e. testing which bats can or cannot discriminate from the habituation, including one control and four test combinations: 1. Rcl<sub>92</sub>-Rcl<sub>92</sub> (control combination; own-own species, same frequencies), 2. Rcl<sub>92</sub>-Rbl<sub>92</sub> (own-foreign species, overlapping frequencies), 3. Rcl<sub>92</sub>-Rbl<sub>87</sub> (own-foreign species, discrete frequencies), 4. Rcl<sub>92</sub>-Rca<sub>87</sub> (own-foreign species, discrete frequencies) and 5. Rca<sub>87</sub>-Rbl<sub>87</sub> (foreign-foreign species, overlapping frequencies). Bats were habituated with the first playback and tested with the second.

The individual call components (Table 3.1) of the playback files (Rcl<sub>92</sub>, Rbl<sub>92</sub>, Rbl<sub>87</sub>, Rca<sub>87</sub>) were measured and analysed to determine the variation between playback categories, as a possible predictor for which components influence discrimination.

### 3.2.2 Experimental Procedure

Habituation – dishabituation experiments were conducted as outlined in section 2.5.1, with video analyses following the procedures outlined in section 2.5.2. Experiments were conducted during daylight hours after the captive bats had been acclimatised to the inverted day-night cycle (section 2.3.2). Each individual was presented with all five playback combinations, including the control and four test combinations. Playback combinations were presented daily over five consecutive days, with each bat presented with a single trial per day.

**TABLE 3.1. LIST OF CALL COMPONENTS MEASURED FOR ANALYSIS OF HD-CF CALLS.**

<b>Component</b>	<b>Abbreviation</b>	<b>Description</b>
Maximum Frequency	$F_{max}$	Maximum frequency reached in the call.
Resting Frequency	RF	Frequency with the highest intensity.
Duration	D	Duration of entire call.
Inter-pulse Interval	IPI	Duration of interval between two calls.
Bandwidth of initial FM	$BW_i$	Range of frequencies represented in the FM component that precedes the CF component of a call.
Bandwidth of terminal FM	$BW_t$	Range of frequencies represented in the FM component that follows the CF component of a call.
Duration of initial FM	$D_i$	Duration of the initial FM component, measured from the start of the call to the point when frequency becomes constant.
Duration of terminal FM	$D_t$	Duration of the terminal FM component, measured from the end of the constant frequency component to the end of the call.
Sweep Rate of Initial FM	$SR_i$	Bandwidth/duration of initial FM component
SR of Terminal FM	$SR_t$	Bandwidth/duration of terminal FM component

### 3.2.3 Statistical Analysis

Behavioural data from habituation-dishabituation experiments were analysed as outlined in section 2.5.3. Call parameters of the playback files were analysed using Discriminant Function Analysis (DFA), followed by Canonical Correlation Analysis to characterise variation between playback classes (Rcl<sub>92</sub>, Rbl<sub>92</sub>, Rbl<sub>87</sub>, Rca<sub>87</sub>). Principal component analysis was used prior to the DFA to extract a set of non-correlated variables from the set of highly correlated echolocation parameters for the DFA.

## 3.3 Results for Species Discrimination Trials

Twelve captive male *R. clivosus* individuals were tested, yielding a total of 49 successful trials (i.e. bats responded to both the onset of habituation and the terminal control). A total of 11 trials were unsuccessful (bats did not respond to either the onset of habituation or the terminal control playback) and were excluded from the analysis. The durations of attentive behaviours were found to be non-normally distributed for all three phases within experimental trials (Shapiro-Wilk: HabSTART  $W = 0.84$ ,  $p < 0.001$ ; HabEND  $W = 0.29$ ,  $p < 0.001$ ; TEST  $W = 0.92$ ,  $p < 0.005$ ), therefore non-parametric tests were used for subsequent analysis. The duration of attentive behavioural responses did not differ significantly between sequential trials for any of the three experimental phases (HabSTART, HabEND, TEST), suggesting that the order in which playback combinations were presented did not influence the bats ability to discriminate between playbacks (Friedman Test: HabSTART  $Fr = 3.86$ ,  $df = 4$ ,  $p > 0.05$ ; HabEND  $Fr = 3.5$ ,  $df = 4$ ,  $p > 0.05$ ; TEST  $Fr = 3.69$ ,  $df = 4$ ,  $p > 0.05$ ).

The duration of attentive behaviours was significantly shorter during the HabEND phase than during the initial onset of HabSTART for all playback combinations (Wilcoxon Pairs Test: Control Rcl<sub>92</sub>-Rcl<sub>92</sub>,  $Z = 2.52$ ,  $p < 0.05$ ; Rcl<sub>92</sub>-Rbl<sub>92</sub>,  $Z = 2.93$ ,  $p < 0.005$ ; Rcl<sub>92</sub>-Rbl<sub>87</sub>,  $Z = 2.52$ ,  $p <$

0.05;  $Rcl_{92}$ - $Rca_{87}$ ,  $Z = 2.93$ ,  $p < 0.005$ ;  $Rca_{87}$ - $Rbl_{87}$ ,  $Z = 2.80$ ,  $p < 0.01$ ), suggesting that habituation was successful.

The ability of bats to discriminate between the habituation and test playback differed significantly between the five playback combinations ( $\chi^2 = 10.91$ ,  $df = 4$ ,  $p < 0.05$ ; Figure 3.1). Post hoc comparisons revealed that in two of the four playback combinations involving foreign species ( $Rcl_{92}$ - $Rbl_{92}$ , Fishers Exact,  $p < 0.0005$ ;  $Rcl_{92}$ - $Rca_{87}$ , Fishers Exact,  $p < 0.05$ ) significantly more individuals respond to the test playback, i.e. discriminated own from foreign calls, than to the control ( $Rcl_{92}$ - $Rcl_{92}$ ) (Figure 3.1). The number of individuals that discriminated the  $Rcl_{92}$ - $Rbl_{87}$  (own-foreign, discrete) combination was not significantly different from that in the control at the 95% confidence level, but was significant at the 94% confidence level (Fishers Exact,  $p = 0.056$ ), with 6 out of 8 bats discriminating the  $Rcl_{92}$ - $Rbl_{87}$  trials. When presented with playbacks from two foreign species with similar frequencies ( $Rca_{87}$ - $Rbl_{87}$ ), only three out of ten individuals discriminated, which did not differ significantly from the control (Fishers Exact,  $p > 0.05$ . Figure 3.1). Thus *R. clivosus* was able to discriminate between con- and heterospecifics, with both overlapping and discrete frequencies, but the ability to discriminate is greatly reduced when presented with similar calls from two foreign species.

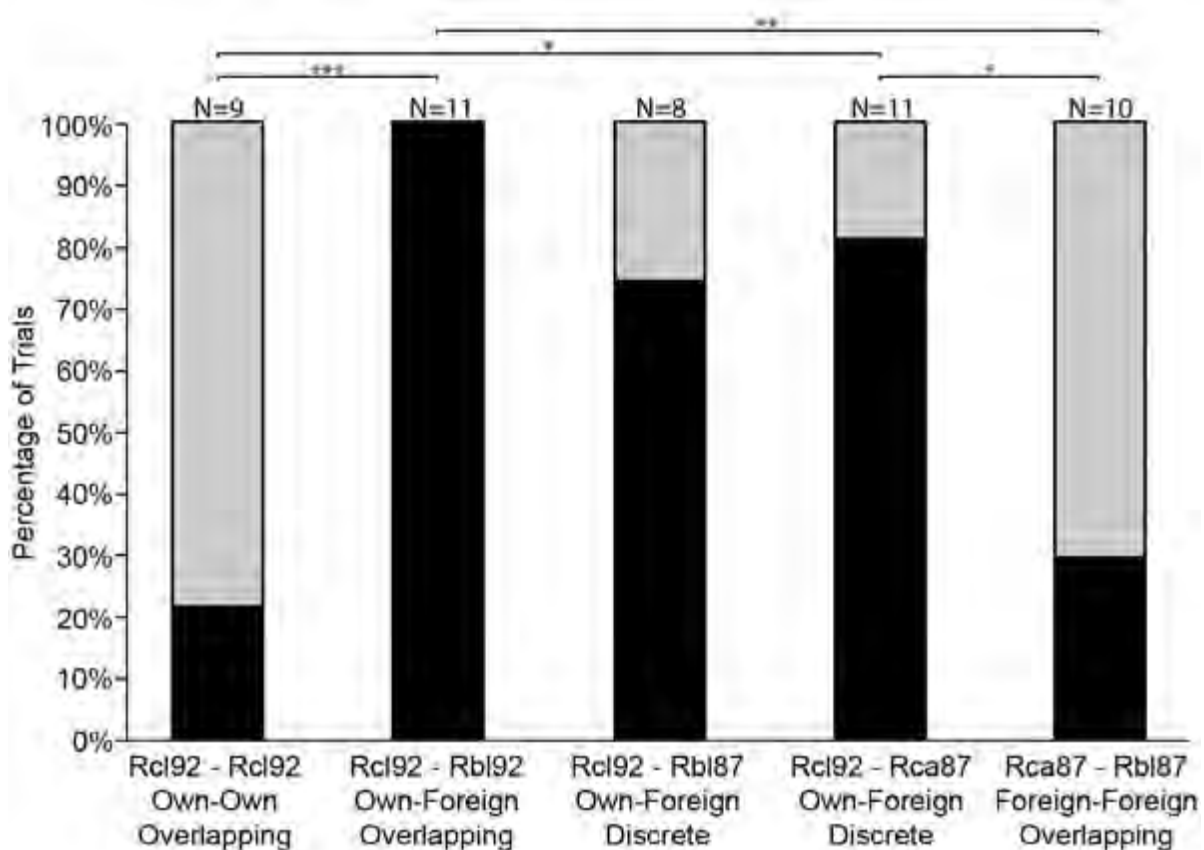
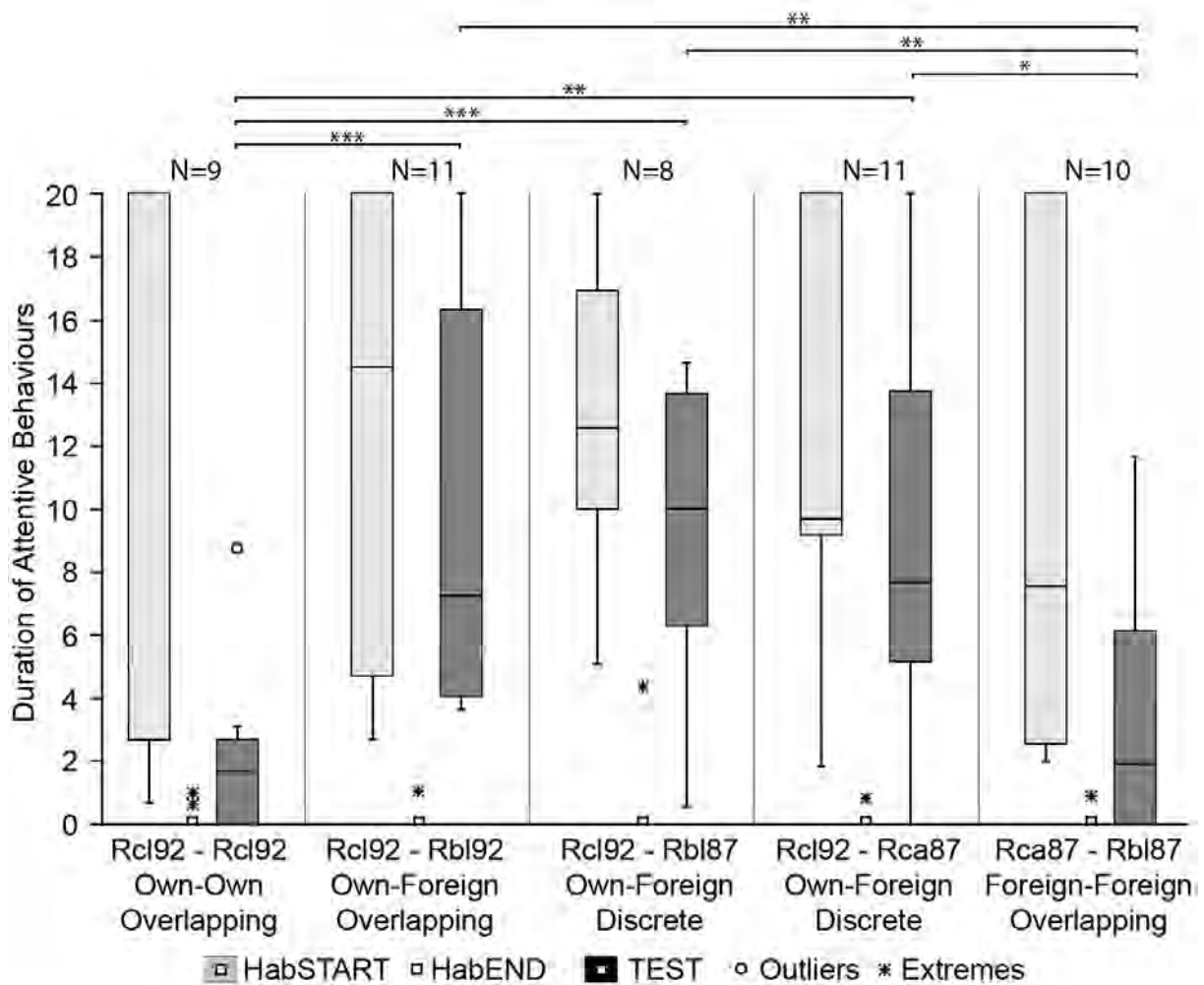


FIGURE 3.1 DISCRIMINATION OF ECHOLOCATION CALLS BY *RHINOLOPHUS CLIVOSUS*. BLACK INDICATES THE PERCENTAGE OF TRIALS IN WHICH THE TEST SUBJECTS DISCRIMINATED BETWEEN HABITUATION AND TEST PLAYBACKS. GREY INDICATES TRIALS IN WHICH TEST SUBJECTS GENERALISED HABITUATION AND TEST PLAYBACKS AS BELONGING TO THE SAME CLASS OF STIMULI. LINES WITH ASTERISKS INDICATE SIGNIFICANT OUTCOMES FROM POST HOC COMPARISONS (FISHER'S EXACT TEST, \*:  $P < 0.05$ , \*\*:  $P < 0.005$ , \*\*\*:  $P < 0.0005$ ). THE NUMBER OF SUCCESSFUL TRIALS FOR EACH COMBINATION IS DISPLAYED ABOVE THE BARS.

The duration of attentive behaviours observed in the TEST phase (Figure 3.2) was significantly longer than that observed in the HabEND phase for all trials involving test playbacks of heterospecifics, regardless of the frequency of the playback (Wilcoxon Pairs Test: Rcl<sub>92</sub>-Rbl<sub>92</sub>,  $Z = 2.93$ ,  $p < 0.005$ ; Rcl<sub>92</sub>-Rbl<sub>87</sub>,  $Z = 2.52$ ,  $p < 0.05$ ; Rcl<sub>92</sub>-Rca<sub>87</sub>,  $Z = 2.66$ ,  $p < 0.01$ ; Rca<sub>87</sub>-Rbl<sub>87</sub>,  $Z = 2.02$ ,  $p < 0.05$ ; Figure 3.2). In contrast, the control playback combination (Rcl<sub>92</sub>-Rcl<sub>92</sub>) showed no significant difference between the HabEND and TEST phases (Wilcoxon Pairs Test, Control,  $Z = 1.78$ ,  $p > 0.05$ ), indicating that the effect of false positive responses by the bats are negligible and that observed behavioural responses are indicative of discrimination between habituation and test playbacks.



**FIGURE 3.2 DURATIONS OF ATTENTIVE BEHAVIOURS DURING THE THREE ANALYSED EXPERIMENTAL PHASES (HABSTART (LIGHT GREY), HABEND (NO BAR) AND TEST (DARK GREY)), WITH BOXES REPRESENTING INTERQUARTILE RANGE AND SQUARES REPRESENTING THE MEDIAN. WHISKERS SHOW NON-OUTLIER RANGE WITHIN 1.5 TIMES THE INTERQUARTILE RANGE. OUTLIERS OUTSIDE OF 1.5 TIMES THE INTERQUARTILE RANGE ARE PRESENTED WITH CIRCLES WITH EXTREMES (OVER 2 TIMES) REPRESENTED BY ASTERISKS. THE DURATIONS OF ATTENTIVE BEHAVIOURS FOR THE HABEND PHASE WERE CLOSE TO ZERO AND IS JUST VISIBLE ON THIS GRAPH. LINES AND ASTERISKS INDICATE OUTCOMES FROM ALL POSSIBLE POST HOC COMPARISONS BETWEEN THE TEST PHASES (\*:  $P < 0.05$ , \*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$ ).**

Furthermore, the duration of attentive behaviours during the TEST playback differed significantly between playback combinations (Kruskal-Wallis,  $H(4) = 18.42$ ,  $p < 0.01$ ). Post-hoc multiple pairwise comparisons between individual playback combinations showed the duration of attentive behaviours in the TEST for all own-foreign combinations (Rcl92-Rbl92, Rcl92-Rbl87 and Rbl92-Rcl87) were significantly higher than for the control, whereas the duration

of attentive behaviours was not significantly higher for the foreign-foreign combination (Figure 3.2).

The longest attentive behavioural responses were to Rcl<sub>92</sub>-Rbl<sub>92</sub> (mean = 10.39 s), followed closely by Rcl<sub>92</sub>-Rbl<sub>87</sub> (mean = 9.34 s) and Rcl<sub>92</sub>-Rca<sub>87</sub> (mean = 8.54 s), although there was no significant difference in response between the three. The control yielded the weakest reactions (mean = 0.73 s), with Rca<sub>87</sub>-Rbl<sub>87</sub> only slightly stronger (mean = 3.29 s), with no significant difference between the two.

#### *Strength of Response for Individual Attentive Behaviours*

Only six of the eight attentive behaviours observed and coded during the HabSTART phase were observed during the TEST phase: RET, SET, FHL, PHL, SC and EC (Table 2.1). Neither partial nor full leg contractions were observed and were therefore excluded from analyses. The distribution of durations and/or frequencies for all six behaviours were not normal (Shapiro-Wilk: All  $p < 0.001$ ), therefore non-parametric tests were used for subsequent tests.

The strength (duration and frequencies) of behavioural response differed between playback combinations for four of the six attentive behaviours (Kruskal-Wallis: RET,  $H(4) = 11.10$ ,  $p < 0.05$ ; SET,  $H(4) = 12.74$ ,  $p < 0.05$ ; PHL,  $H(4) = 12.84$ ,  $p < 0.05$ ; SC,  $H(4) = 13.74$ ,  $p < 0.01$ ). The duration and frequency of the two remaining attentive behaviours (FHL and EC) did not differ significantly between playback combinations at the 95% confidence level (Kruskal-Wallis: FHL,  $H(4) = 8.61$ ,  $p > 0.05$ ; EC,  $H(4) = 7.09$ ,  $p > 0.05$ ).

Post hoc tests found no significant difference between individual playback combinations for any of the attentive behaviours with two exceptions: the duration of Slow Ear Twitching was significantly higher for the Rcl<sub>92</sub>-Rbl<sub>92</sub> playbacks than for the control and the duration

of Partial Head Lift was significantly higher for Rcl92-Rbl87 playback combination than for the Rca87-Rbl87 playback combination.

### *Playback Call Analyses*

Examination of a correlation matrix revealed significant correlations between all but three call parameter pairings (Pearson product-moment correlation: N=932,  $p < 0.05$ ). Principal component analyses (PCA) was therefore used to extract a set of ten linearly uncorrelated variables from variables measured for 926 calls across the four test classes used as playbacks. The first four Principal components (PC 1-4) had Eigenvalues  $\geq 1$  and accounted for 79.23% of the variation and were used for further analysis (Table 3.2).

**TABLE 3.2 PRINCIPAL COMPONENT LOADINGS FOR THE FIRST FOUR PRINCIPAL COMPONENTS EXTRACTED FROM THE TEN ECHOLOCATION MEASUREMENTS. NUMBERS IN BOLD INDICATE COMPONENTS WITH THE STRONGEST LOADINGS FOR EACH VARIABLE.**

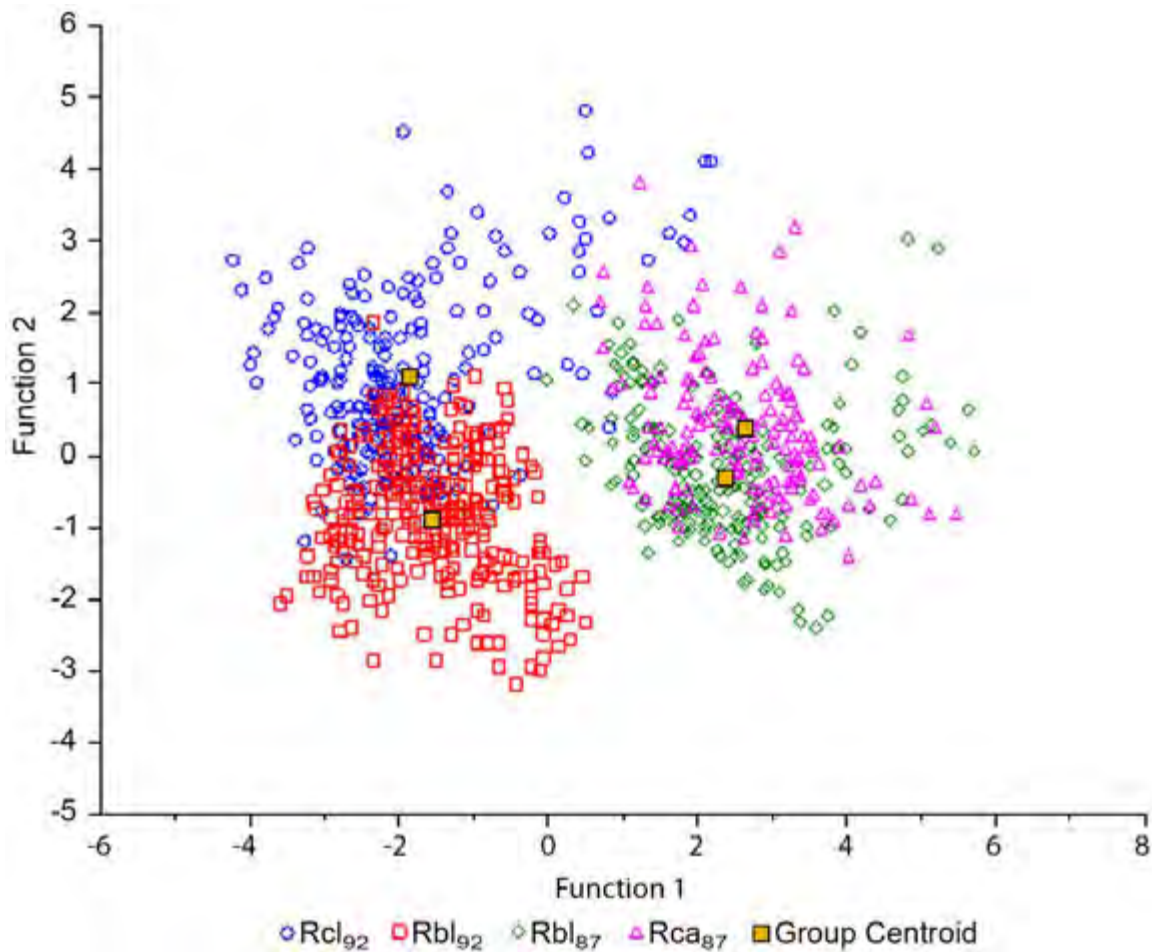
Variable	PC 1	PC 2	PC 3	PC 4
Duration	-0.015	<b>-0.762</b>	0.342	0.073
Inter-Pulse-Interval	0.264	<b>-0.624</b>	0.346	-0.182
Peak Frequency	<b>-0.641</b>	0.424	0.449	0.320
Maximum Frequency	-0.436	0.530	<b>0.633</b>	0.185
Bandwidth of initial FM	<b>-0.754</b>	-0.531	0.025	-0.178
Bandwidth of terminal FM	<b>-0.755</b>	-0.009	-0.463	0.227
Duration of initial FM	<b>-0.642</b>	-0.633	-0.031	-0.114
Duration of terminal FM	-0.047	-0.538	-0.183	<b>0.766</b>
Sweep Rate Initial FM	<b>-0.663</b>	-0.146	0.157	-0.291
Sweep Rate Terminal FM	<b>-0.677</b>	0.413	-0.328	-0.210

The bandwidths of the initial and terminal FM components ( $BW_i$  and  $BW_t$ ), as well as the sweep rate in these two components ( $SR_i$  and  $SR_t$ ) loaded highest on PC1. Call duration ( $D$ ), interpulse interval (IPI) and the duration of the initial FM component ( $D_i$ ) loaded highest on PC2. Peak and maximum frequencies ( $RF$  and  $F_{max}$ ) as well as the bandwidth of the terminal FM component ( $BW_t$ ) loaded highest on PC3. The duration of the terminal FM component ( $D_t$ ) loaded highest on PC4.

The values resulting from the PCA did not follow a normal distribution (Shapiro-Wilk:  $W = 0.99$ ,  $p < 0.05$ ), however the DFA is fairly robust to deviation from normality (Tabachnick and Fidell, 1996) and the results can still be considered trustworthy. Discriminant function analysis (DFA; Figure 3.3) showed significant separation between the four playback groups based on their acoustic characteristics (Wilks  $\lambda = 0.102$ ,  $F_{12,2437} = 277.37$ ,  $p < 0.001$ ) and all four playback combinations differed significantly from one another (Table 3.3). The first discriminant function (Function 1) explained 85.1% of the variance and Function 2 explained a further 10.8% (Figure 3.3). Calls for the four phonic types were correctly classified into their phonic types ( $Rcl_{92}$ ,  $Rbl_{92}$ ,  $Rbl_{87}$  and  $Rca_{87}$ ) on the basis of their acoustic parameters with a classification success rate of  $>80\%$ . However,  $Rca_{87}$  was an exception with a lower classification success of 65%. Function 1 contained high loadings of PC1 and PC3 and its discriminatory power is therefore mainly characterised by spectral parameters ( $RF$ ,  $F_{max}$ ,  $BW_i$  and  $BW_t$ ) and sweep rate ( $SR_i$  and  $SR_t$ ). Function 2 which had much lower discriminatory power contained PC2 and PC4 and thus is characterised by temporal parameters ( $D$ ,  $D_i$ ,  $D_t$ , and IPI).

**TABLE 3.3 F-VALUES OBTAINED FROM DISCRIMINANT FUNCTION ANALYSIS ON ECHOLOCATION CALL PARAMETERS OF FOUR PLAYBACK CATEGORIES (DF=4; ALL P<0.01).**

Species	Rcl92	Rbl92	Rbl87	Rca87
Rcl92		123.4102	556.7822	513.3864
Rbl92	123.4102		527.8203	515.6454
Rbl87	556.7822	527.8203		53.3321
Rca87	513.3864	515.6454	53.3321	



**FIGURE 3.3 PLOT OF THE FIRST TWO FUNCTIONS OF A DFA ON TEN ACOUSTIC PARAMETERS OF PLAYBACK STIMULI. EACH CALL OF EACH PLAYBACK CLASS IS PLOTTED (N = 926) AND EACH PLAYBACK CLASS IS COLOUR CODED.**

Accordingly, the graphical plot of those two functions reveals the four playback classes formed two primary clusters separated along Function 1, i.e. spectral parameters (Figure 3.3). One primary cluster (on the right half of Figure 3.3) was comprised of calls from the two 87-kHz classes (Rbl<sub>87</sub>, Rca<sub>87</sub>) and the other primary cluster was comprised of calls from the two

92-kHz classes (Rcl<sub>92</sub>, Rbl<sub>92</sub>) with Mahalanobis distances > 16.4 (Table 3.4). There was hardly any separation of the two classes of calls within the 87 kHz cluster along Function 1 and Function 2 (Figure 3.3). Calls within this cluster differed by a Mahalanobis distance between their centroids of only 2.3 and reflects the low classification success of Rca87 calls. Calls within the 92-kHz cluster were separated along Function 2, i.e. temporal parameters and differed by Mahalanobis distance of 3.7, over 50% more than the 87 kHz cluster. This within-cluster separation occurred mainly along Function 2, attributing the variation to temporal call components.

**TABLE 3.4 SQUARED MAHALANOBIS DISTANCES OBTAINED FROM DISCRIMINANT FUNCTION ANALYSIS ON ECHOLOCATION CALL PARAMETERS OF FOUR PLAYBACK CATEGORIES.**

Species	Rcl92	Rbl92	Rbl87	Rca87
Rcl92		3.70555	20.28414	21.79342
Rbl92	3.70555		16.41535	19.14054
Rbl87	20.28414	16.41535		2.32124
Rca87	21.79342	19.14054	2.32124	

### 3.4 Discussion – Species Discrimination by *R. clivosus* using echolocation

This study provides additional evidence that horseshoe bats can distinguish conspecific echolocation calls from those of heterospecifics. Bats habituated and tested with conspecific echolocation calls showed little response to the change in playback, suggesting that they correctly generalised both playbacks as belonging to the same class (this was the control). In contrast, bats habituated with conspecific calls and tested with calls from a foreign species showed behavioural responses to the change in playback, demonstrating the ability to discriminate heterospecific calls from those of conspecifics. This corroborates the findings of previous studies (Schuchmann and Siemers, 2010a; Li et al., 2014; Bastian and Jacobs, 2015)

that echolocating bats discriminate between species based purely on echolocation. However, as with previous studies (Schuchmann and Siemers, 2010a; Bastian and Jacobs, 2015), discrete frequency bands were not essential for discrimination of foreign species. *Rhinolophus clivosus* were able to discriminate calls from *R. blasii* echolocating at frequencies that overlapped entirely with those of conspecific calls ( $Rcl_{92}$  –  $Rbl_{92}$ ). It is therefore likely that multiple components of echolocation, in addition to RF, are used by *R. clivosus* for species identification.

For successful discrimination, bats require some level of separation in acoustic space (Bastian and Jacobs, 2015). There is therefore a reduction in discriminatory ability when variation in acoustic space is limited. This limitation is demonstrated by the inability of *R. clivosus* to discriminate between  $Rca_{87}$  and  $Rbl_{87}$ , two heterospecifics with playbacks that overlap strongly in acoustic space (Figure 3.1). Calls within these two classes showed very little separation in acoustic space across both temporal and spectral call parameters. The overlap in acoustic space between these two sets of calls may reduce the ability of *R. clivosus* to discriminate between  $Rca_{87}$  and  $Rbl_{87}$ . Individually, however, both  $Rca_{87}$  and  $Rbl_{87}$  show pronounced separation in spectral parameters from the calls of the  $Rcl_{92}$ , and listening *R. clivosus* could readily discriminate these calls from those of their own species.

In contrast, the calls of  $Rbl_{92}$  were strongly discriminated from those of  $Rcl_{92}$ , despite their overlapping frequencies. However, unlike the calls of  $Rca_{87}$  and  $Rbl_{87}$  which shared much of the same acoustic space in terms of both spectral and temporal parameters,  $Rbl_{92}$  and  $Rcl_{92}$  were separated to some degree by the temporal parameters of call duration, duration of the initial and terminal FM components and the inter-pulse interval. The separation in temporal parameters likely provides the variation in acoustic space required for effective recognition.

The strength of the discrimination between Rcl92 and Rbl92 was a key difference between this study and that of Schuchmann and Siemers (2010a). The ability of *R. mehelyi* and *R. euryale* to discriminate foreign calls when the frequencies of those calls overlapped with their own was reduced relative to their discrimination of calls from foreign species with discrete frequency bands. In contrast, there was no significant difference in the discrimination performance of *R. clivosus* between combinations of either discrete or overlapping call frequencies (Figure 3.1). In fact, the opposite was observed, the level of discrimination was the highest for the Rcl92–Rbl92 trials. It is worth noting that we do not know the level of separation in acoustic space between *R. mehelyi* and *R. euryale* as examined by Schuchmann & Siemers (2010a) and therefore do not know if the discrimination ability of these species is affected by their separation in acoustic space beyond resting frequency,

The strong discrimination performance observed in the Rcl92–Rbl92 trials further supports the notion that components of echolocation other than RF may provide species specific signatures which can be used by bats for species discrimination. The stronger performance may be related to the bats paying greater attention to these additional components when frequencies are similar, provided these additional components are sufficiently different to facilitate discrimination.

The similarity in calls of Rca87 and Rbl87 may be due to signal convergence in allopatry. Considering that bat echolocation functions primarily in orientation and foraging, the parameters of echolocation are strongly shaped by the habitat and environment in which a species has evolved (Neuweiler, 1989). As a result, bats of a similar size, habitat use and foraging strategy have been shown to converge on similar echolocation call structures (Schnitzler and Kalko, 2001; Fenton et al., 2004). *Rhinolophus capensis* and *R. blasii* are not

sympatric anywhere in their ranges (Figure 2.3), thus there is no selective pressure to maintain 'private frequency bands' (Heller and Von Helversen, 1989) in order to facilitate communication. In the absence of competition for acoustic space between *R. capensis* and *R. blasii*, similarities in their foraging ecology and habitat (Monadjem et al., 2010) likely result in the evolutionary convergence of both species on the same acoustic space in allopatry without compromising the communication potential in either species.

Similarly, signal convergence in allopatry together with divergence in sympatry, mediated by evolution of unambiguous acoustic signals, may explain the range of signals used by *R. blasii*. The distributions of *R. clivosus* and *R. blasii* overlap in several areas across their ranges (Monadjem et al., 2010), with variation in RF observed across their distributions. In sympatric areas of southern Africa, *R. blasii* uses the lower 87 kHz frequency band, whereas in allopatric regions, *R. blasii* echolocates in the higher 92 kHz frequency band, the same as that used by *R. clivosus* (this study). This suggests that in sympatry, competition for acoustic space may have resulted in extensive shifts in spectral parameters (particularly in RF) and smaller shifts in temporal parameters (Figure 3.3). However, in allopatry, where *R. blasii* is released from potential competition with *R. clivosus* for acoustic space, there is convergence in its RF with that of *R. clivosus* but with sufficient differences between the temporal components to allow discrimination.

If competition is responsible for the divergence in calls between sympatric *R. blasii* and *R. clivosus* it provides support for the view that where signal space is available, misclassification as a result of overlapping signal parameters may be minimized through the evolution of divergent signals (Bastian and Jacobs, 2015). Again it becomes important to consider the constraints on signal divergence due to the orientation/foraging functions of

echolocation. An evolutionary trade-off exists between maintaining an optimal call design for orientation and maintaining private acoustic space to facilitate communication. Moreover, there are morphological and physiological constraints on echolocation that need to be overcome for a signal to diverge. Apart from the direct effect of body size on the production of sound- as body size increases, peak echolocation frequency decreases (Novick, 1977; Heller and Von Helversen, 1989; Barclay and Brigham, 1991)- the frequency range available for echolocation calls is constrained by the acoustic fovea in horseshoe bats. The acoustic fovea refers to a disproportionate representation for specific frequencies in the auditory system of horseshoe bats (Schuller and Pollak, 1979). The increased perceptual acuity allowed for by the acoustic fovea is so great that bats shift the frequency of emitted calls to compensate for Doppler shift during flight to ensure that the Doppler shifted frequency of the returning echo falls within the range of the acoustic fovea (Schnitzler and Denzinger, 2011). Divergence in echolocation calls to accommodate a communicative function would need to account for these morphological and physiological constraints, ultimately resulting in a physiological change. Given these constraints which favour a stable system and the strong ability of bats to discriminate between calls with both discrete and overlapping frequencies, communication offers a viable explanation for the level of signal divergence observed in many species of rhinolophid, including *R. blasii*.

## Chapter 4

### Echolocation Components Influencing Communication

#### 4.1 Introduction

A potential role of multiple components of echolocation, beyond that of resting frequency alone, in communication has been suggested by a number of authors (Schuchmann and Siemers, 2010a; Bastian and Jacobs, 2015). The notion derives from observations that in cases where two species echolocation calls overlap in resting frequency, listening bats are often still able to discriminate between their echolocation calls. Schuchmann & Siemers (2010a) were the first to observe that *R. mehelyi* and *R. euryale* were both able to discriminate calls of another species with similar frequency to their own calls, suggesting that call features other than resting frequency play a role in communication. They proposed the terminal FM component to be a potential candidate for purveying species specific information.

Bastian and Jacobs (2015) demonstrated that certain populations of *R. capensis* were also able to discriminate calls from other horseshoe bat species with overlapping frequencies. They suggest that separation in acoustic space, accounting for all aspects of echolocation and not just frequency, may aide in the ability of bats to discriminate between the echolocation calls of different species. Furthermore, they expand on the suggestion by Schuchmann & Siemers (2010a) that the terminal FM component may influence communication, expanding to include namely call duration, duration to maximum intensity, minimum frequency of final FM component and call entropy as potentially important components in echolocation. These findings led to the reformulation of the Acoustic Communication Hypothesis to include multidimensional acoustic space, rather than just frequency, which may be partitioned in an

assemblage of sympatric congeneric species so that each species occupies a distinct acoustic space facilitating intraspecific communication and species discrimination. Although Schuchmann & Siemers (2010a) and Bastian & Jacobs (2015) speculated that additional components of echolocation calls may serve as discrimination cues in a communication context, with Bastian & Jacobs (2015) going on to examine which components show significant variation to influence communication, no study has experimentally demonstrated the ability of bats to detect changes in parameters of individual call components.

The case for the ACH and echolocation as a means of communication can be strengthened if the discrimination echolocation allows is used by the bats to enhance their survival or reproductive success. For this to be the case, echolocation would need to function in mate choice, resulting in sexual selection of traits associated with a higher reproductive success. I discuss the intricacies of echolocation and mate choice in detail in Chapter 5, but for now I will touch on the notion that body condition can be used as an indicator of fitness, including aspects of foraging success, fighting ability and resilience to environmental factors (Jakob et al., 1996), which in turn impact on reproductive success. Body condition index (BCI) is a measure of the condition of an individual based on a measure of weight when size is standardised. Puechmaile *et al.* (Puechmaile et al., 2014) found a strong link between echolocation frequency and BCI in the horseshoe bat *R. mehelyi*. They then proceeded to experimentally demonstrate that female *R. mehelyi* preferentially selected males with a higher BCI from their echolocation calls, giving behavioural evidence for the role of echolocation in mate choice in bats. Their study, however, used echolocation call frequency as a proxy for body condition, and did not directly test the ability of bats to discriminate the calls of individuals with varying body conditions.

In this chapter I investigated, firstly, which acoustic components of the call in addition to frequency may carry discrimination cues and secondly, whether horseshoe bats can use this discriminatory ability to identify potential cues for reproductive fitness, using BCI as an indicator of fitness.

$H_1$ : *R. clivosus* can discriminate between echolocation calls based on individual components of echolocation besides frequency.

$H_0$ : *R. clivosus* does not discriminate between echolocation calls which overlap in frequency despite varying in other components.

Considering the previous investigations of Schuchmann and Siemers (2010a) and Bastian and Jacobs (2015), one would predict that the FM components and temporal components likely act as communication cues.

## 4.2 Methods for Call Component Trials

### 4.2.1 Playback File Preparation

To explore which call components have the potential to encode discrimination cues, we used playback sequences compiled from fully synthetic calls, in which we varied one acoustic component at a time while keeping all others constant. I used mean values measured from natural *R. clivosus* calls [N=20 (10 males; 10 females); n=10 per individual; location: De Hoop Nature Reserve, Table 4.1] for the components RF, bandwidth, sweep rate of FM components, duration of CF component and IPI to produce a synthetic average call (designated “Control call”).

**TABLE 4.1 MEAN VALUES OF ECHOLOCATION PARAMETERS FOR *RHINOLOPHUS CLIVOSUS* AT DE HOOP NATURE RESERVE.**

		RF	D	BW <sub>i</sub>	D <sub>i</sub>	SR <sub>i</sub>	BW <sub>t</sub>	D <sub>t</sub>	SR <sub>t</sub>	IPI
Female	Mean	91.93	34.51	11.15	1.94	5.90	22.71	3.10	8.10	81.07
	Std Dev	0.87	6.28	4.11	0.87	1.25	2.96	1.10	2.45	46.45
Male	Mean	91.88	34.83	12.00	2.16	5.70	22.66	3.06	7.80	107.36
	Std Dev	0.86	8.46	4.74	0.95	1.47	5.03	0.87	2.36	57.79

Based on this average call template I created six call variants each differing in only one specific call parameter, i.e. either frequency of the CF component or sweep rate of the FM component or duty cycle. The altered parameter values were based on mean minimum and maximum values calculated from measurements taken from the natural calls. The call variant in which the frequency of the CF component was altered (designated “Frequency”) was created by decreasing the frequencies of the CF component of the Control call by 5 kHz. Call variants designated “FM1” and “FM2” were compiled by changing the sweep rate of the flanking FM components of the Control call. Thus FM1 had a lower sweep rate and FM2 a higher sweep rate than the average of the natural calls. Both the initial and terminal FM components were altered in the same way for each of FM1 and FM2. The call variants “Duty Cycle1” and “Duty Cycle2” were compiled by altering the duration of the CF component of the calls. In Duty Cycle1 the duration of the CF component was set to the maximum naturally observed duration, decreasing the period and increasing the duty cycle in sequences of these calls. Accordingly, in Duty Cycle2, the durations of the CF components were set to the minimum naturally observed duration increasing the period of the calls and lowering the duty cycle in sequences of these calls.

Playback files were compiled by repeating a single call variant (Control, Frequency, FM1, FM2, Duty Cycle1, Duty Cycle2), maintaining an IPI equal to the average of naturally recorded

intervals, until the required duration of the playback file was reached (habituation playbacks: 300 s; test playbacks: 20 s). The playback files were assigned to four playback combinations each containing a habituation playback of 300 s and a test playback of 20 s: 1: Control – Control, 2. Control – Frequency, 3. FM1 – FM2, 4. Duty Cycle1 – Duty Cycle2.

The experiment was then extended to investigate if echolocation has the potential to be used by bats in mate choice. We tested if bats are able to discriminate between echolocation calls from low or high body condition males. In addition to the synthetically produced and altered signals described above, I produced two playbacks using calls from male *R. clivosus* individuals (N=6) with extremes of either high or low BCI scores. BCI was calculated using the ratio between mass and forearm length. The echolocation call recordings of the top and bottom three individuals in terms of BCI were used to create the high and low BCI playbacks, respectively, using 20 high quality calls per individual. Semi-synthetic duplicates of each call were created and compiled following the same procedures as described in 3.2.1. The BCI playbacks were assigned to a fifth playback combination (5. High BCI – Low BCI) and were included into the playback series along with the four combinations described above.

#### 4.2.2 Experimental Procedure

Habituation – dishabituation experiments were conducted as outlined in section 2.5.1, with video analyses following the procedures outlined in section 2.5.2. Experiments were conducted after sunset, and run at night to align with the nocturnal nature of the bats. Each individual was presented with all five playback combinations listed above, including the control and four test combinations. Playback combinations were presented across two consecutive nights, with each bat presented with either two or three trials per night.

### 4.2.3 Statistical Analysis

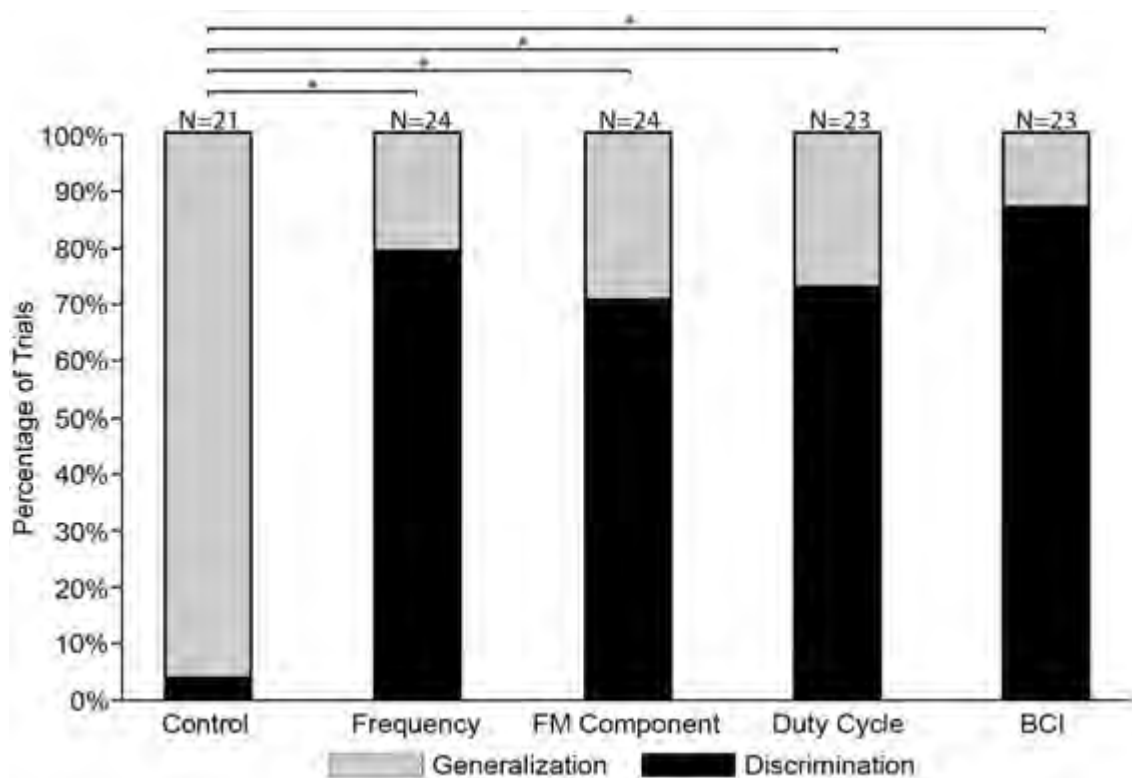
Behavioural data from habituation-dishabituation experiments were analysed as outlined in section 2.5.3. Call parameters of the playback files were analysed using Discriminant Function Analysis (DFA), followed by Canonical Correlation Analysis to characterise variation between playback classes (Male – Female and High BCI – Low BCI). Principal component analysis was used prior to the DFA to extract a set of uncorrelated variables from the set of highly correlated echolocation parameters. DFAs are strongly influenced by correlated variables (Klecka, 1980).

## 4.3 Results for Call Component Trials

Thirty-one *R. clivosus* individuals were tested (10 males, 21 females) for the five playback combinations, yielding a total of 119 successful trials. A total of 36 trials were unsuccessful (the bat did not respond to either the onset of habituation or the terminal control signal) and were excluded from the analysis. The behavioural responses of males and females to the test playbacks did not significantly differ and results were thus pooled ( $\chi^2 = 0.0003$ ,  $df = 1$ ,  $p > 0.05$ ). The durations of attentive behaviours were found to be non-normally distributed for all analysed phases (Shapiro-Wilk: HabSTART  $W = 0.88$ ,  $p < 0.0001$ ; HabEND  $W = 0.13$ ,  $p < 0.0001$ ; TEST  $W = 0.79$ ,  $p < 0.0001$ ). The strength of behavioural responses did not differ significantly between sequential trials for any of the three experimental phases, suggesting that the order in which playback combinations were presented to an individual did not influence the bats ability to discriminate between playbacks (Friedman Test: HabSTART  $Fr = 3.23$ ,  $df = 4$ ,  $p > 0.05$ ; HabEND  $Fr = 4$ ,  $df = 4$ ,  $p > 0.05$ ; TEST  $Fr = 4.07$ ,  $df = 4$ ,  $p > 0.05$ ). The successful habituation to the habituation stimulus was confirmed by significantly lower durations of attentive behaviours during the HabEND phase than during the HabSTART for all

playback combinations (Wilcoxon Pairs Test: Control,  $Z = 4.01$ ,  $p < 0.001$ ; Frequency,  $Z = 4.29$ ,  $p < 0.001$ ; FM,  $Z = 4.20$ ,  $p < 0.001$ ; Duty Cycle,  $Z = 4.20$ ,  $p < 0.001$ ; BCI,  $Z = 4.20$ ,  $p < 0.001$ ).

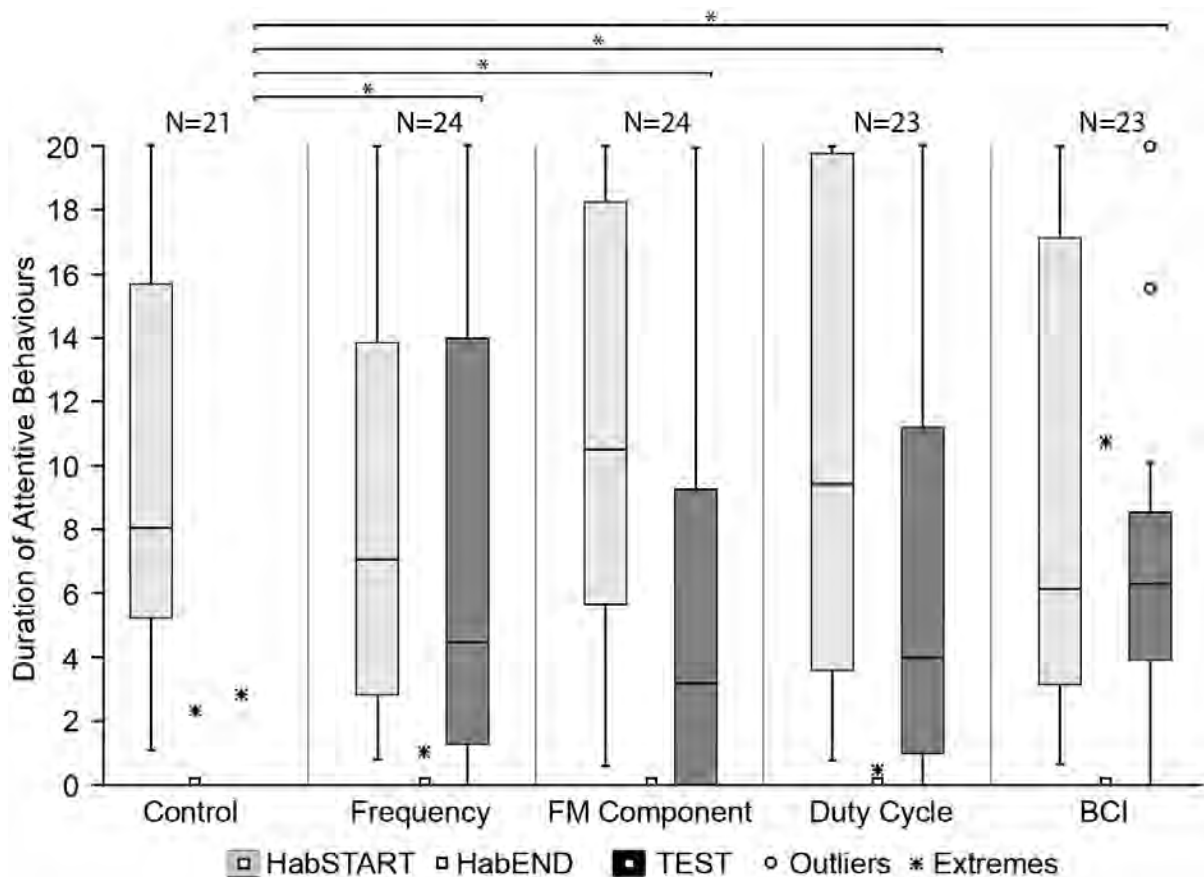
The ability of bats to discriminate between the habituation and test playbacks differed significantly between the five playback combinations ( $\chi^2 = 42.46$ ,  $df = 4$ ,  $p < 0.001$ ; Figure 4.1). Post hoc pairwise comparisons revealed that significantly more individuals discriminated the test playback from the habituation playback for all four test playback combinations than for the control (Figure 4.1). There was no significant difference in the number of instances of discrimination between any of the other playback combinations (Frequency, FM Component, Duty Cycle, BCI).



**FIGURE 4.1 DISCRIMINATION BY *RHINOLOPHUS CLIVOSUS* INDIVIDUALS BETWEEN PLAYBACKS OF INDIVIDUALLY CONTROLLED ECHOLOCATION COMPONENTS. BLACK INDICATES THE PERCENTAGE OF TRIALS IN WHICH THE TEST SUBJECTS DISCRIMINATED BETWEEN HABITUATION AND TEST PLAYBACKS. GREY INDICATES THE PERCENTAGE OF TRIALS IN WHICH TEST SUBJECTS GENERALISED HABITUATION AND TEST PLAYBACKS AS BELONGING TO THE SAME CLASS OF STIMULI. LINES AND ASTERISKS INDICATE OUTCOMES FROM ALL POSSIBLE POST HOC COMPARISONS (FISHER'S EXACT TEST, \*:  $P < 0.0001$ ). THE NUMBER OF SUCCESSFUL TRIALS FOR EACH COMPONENT IS DISPLAYED ABOVE THE BARS.**

The control playback combination showed no significant difference in the duration of attentive behaviours between the HabEND and TEST phases (Wilcoxon Pairs Test, Control,  $Z = 0.54$ ,  $p > 0.05$ ), indicating that the observed behavioural responses are indicative of discrimination between habituation and test playbacks. All four test combinations yielded a significant increase in attentive behaviours in response to the test playback (Wilcoxon Pairs Test, Frequency:  $Z = 3.72$ ,  $p < 0.001$ ; FM:  $Z = 3.52$ ,  $p < 0.005$ ; Duty Cycle:  $Z = 3.12$ ,  $p < 0.001$ ; BCI:  $Z = 3.92$ ,  $p < 0.001$ ).

The duration of attentive behaviours in response to the test playback differed significantly between playback combinations (Kruskal-Wallis,  $H(4) = 31.52$ ,  $p < 0.001$ ). Post-hoc multiple pairwise comparisons between individual playback combinations showed the duration of behavioural reaction was significantly higher than the control for all four test combinations (Figure 4.2). There was however no significant difference between any of the four test combinations (Figure 4.2).



**FIGURE 4.2 DURATIONS OF ATTENTIVE BEHAVIOURS DURING THE THREE ANALYSED PHASES (HABSTART (LIGHT GREY), HABEND (NO BAR) AND TEST (DARK GREY)), WITH BOXES REPRESENTING INTERQUARTILE RANGE. WHISKERS SHOW NON-OUTLIER RANGE WITHIN 1.5 TIMES THE INTERQUARTILE RANGE. OUTLIERS OUTSIDE OF 1.5 TIMES THE INTERQUARTILE RANGE ARE PRESENTED WITH CIRCLES WITH EXTREMES (OVER 2 TIMES) REPRESENTED BY ASTERISKS. THE DURATIONS OF ATTENTIVE BEHAVIOURS FOR THE HABEND PHASE WERE CLOSE TO ZERO AND ARE BARELY VISIBLE ON THIS GRAPH. LINES AND ASTERISKS INDICATE STATISTICAL OUTCOMES FROM ALL POSSIBLE POST HOC COMPARISONS (\*:  $P < 0.01$ ).**

The longest attentive behavioural reactions were to Frequency (Average = 7.7 s), followed closely by Duty Cycle (Average = 6.68 s), BCI (Average = 6.39 s), FM Components (Average = 5.18 s), although there was no significant difference in response between the four. The control yielded the weakest reactions (Average = 0.15 s).

*Rhinolophus clivosus* also successfully discriminated between the two sets of conspecific calls representing either high or low BCI males, with the rate of discrimination being significantly higher than for the control both in terms of number of successful trials (Figure 4.1) and the duration of attentive behaviours (Figure 4.2).

### *Strength of Response for Individual Attentive Behaviours*

All eight attentive behaviours coded for during analyses were observed during the TEST phase. The distribution of durations/frequencies for all eight behaviours were not normal (Shapiro-Wilk: All  $p < 0.001$ ), therefore non-parametric tests were used for subsequent tests.

The strength (duration and/or frequency) of behavioural response differed between playback combinations for four of the eight attentive behaviours (Kruskal-Wallis: SET,  $H(4) = 23.40$ ,  $p < 0.005$ ; PHL,  $H(4) = 9.62$ ,  $p < 0.05$ ; SC,  $H(4) = 22.43$ ,  $p < 0.001$ ; EC,  $H(4) = 16.33$ ,  $p < 0.005$ ).

The duration of response for the remaining four attentive behaviours (RET, PLC, FLC and FHL) did not differ significantly between playback combinations at the 95% confidence level (Kruskal-Wallis: RET,  $H(4) = 8.08$ ,  $p > 0.05$ ; PLC,  $H(4) = 3.03$ ,  $p > 0.05$ ; FLC,  $H(4) = 3.79$ ,  $p > 0.05$ ; FHL,  $H(4) = 2.20$ ,  $p > 0.05$ ). However, Rapid Ear Twitch was significant at a 91% confidence interval, suggesting that although statistically insignificant, this behaviour may still be biologically significant indicators of discrimination in bats.

### *Playback Call Analyses*

Examination of a correlation matrix revealed significant correlations between all but three call parameter pairings (Pearson product-moment correlation:  $N=1192$ ,  $p < 0.05$ ). Principal component analyses (PCA) was therefore used to extract a set of ten linearly uncorrelated variables from variables measured for 1192 calls across the four test classes used as playbacks. The first four Principal components (PC 1-4) had Eigenvalues  $\geq 1$  and accounted for 71.23% of the variation and were used for further analysis (Table 4.2).

**TABLE 4.2 PRINCIPAL COMPONENT LOADINGS FOR THE FIRST FOUR PRINCIPAL COMPONENTS EXTRACTED FROM THE TEN ECHOLOCATION MEASUREMENTS. NUMBERS IN BOLD INDICATE COMPONENTS WITH THE STRONGEST LOADINGS ON EACH PRINCIPAL COMPONENT.**

Variable	PC 1	PC 2	PC 3	PC 4
Duration	<b>0.782</b>	0.229	0.099	0.247
Inter-Pulse-Interval	<b>0.687</b>	0.078	-0.059	0.510
Peak Frequency	-0.152	0.249	<b>0.571</b>	0.061
Maximum Frequency	-0.082	-0.035	<b>-0.600</b>	0.451
Bandwidth of initial FM	<b>0.666</b>	0.533	0.070	-0.070
Bandwidth of terminal FM	-0.175	0.392	<b>0.660</b>	-0.046
Duration of initial FM	0.079	-0.395	0.458	<b>0.620</b>
Duration of terminal FM	0.569	<b>-0.613</b>	0.268	-0.308
Sweep Rate Initial FM	0.496	<b>0.604</b>	-0.229	-0.319
Sweep Rate Terminal FM	-0.581	<b>0.695</b>	0.009	0.300

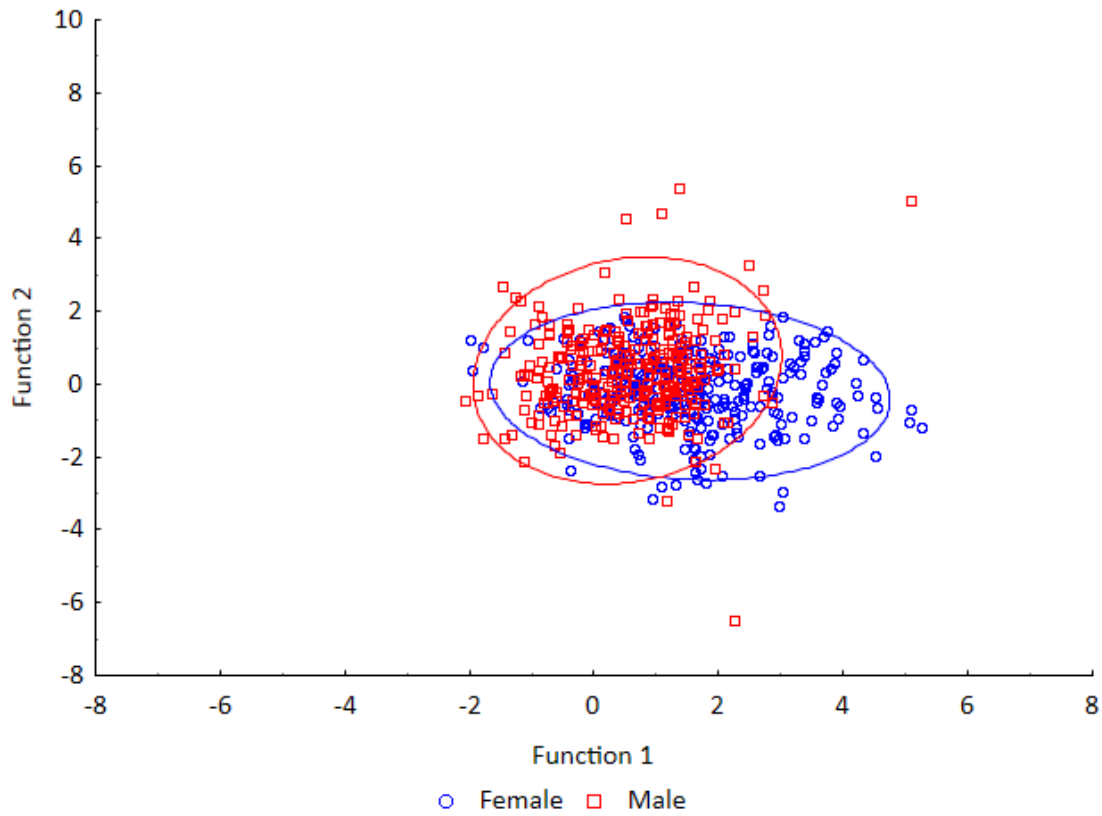
Call duration ( $D$ ), interpulse interval ( $IPI$ ) and the bandwidth of the initial FM component ( $BW_i$ ) loaded highest on PC1. The duration of the terminal FM component ( $D_t$ ), together with the sweep rates of both the initial and terminal FM components ( $SR_i$  and  $SR_t$ ) loaded highest on PC2. Peak and maximum frequencies ( $RF$  and  $F_{max}$ ) as well as the bandwidth of the terminal FM component ( $BW_t$ ) loaded highest on PC3. The duration of the initial FM component ( $D_i$ ) loaded highest on PC4.

As in Chapter 3, the values resulting from the PCA did not follow a normal distribution (Shapiro-Wilk:  $W = 0.99$ ,  $p < 0.05$ ), however the DFA is robust to deviations from normality (Tabachnick and Fidell, 1996). Discriminant function analysis (DFA; Figure 3.3) showed significant separation between the four playback groups based on their acoustic characteristics (Wilks  $\lambda = 0.6342$ ,  $F_{12,3135} = 49.08$ ,  $p < 0.001$ ) and all four playback

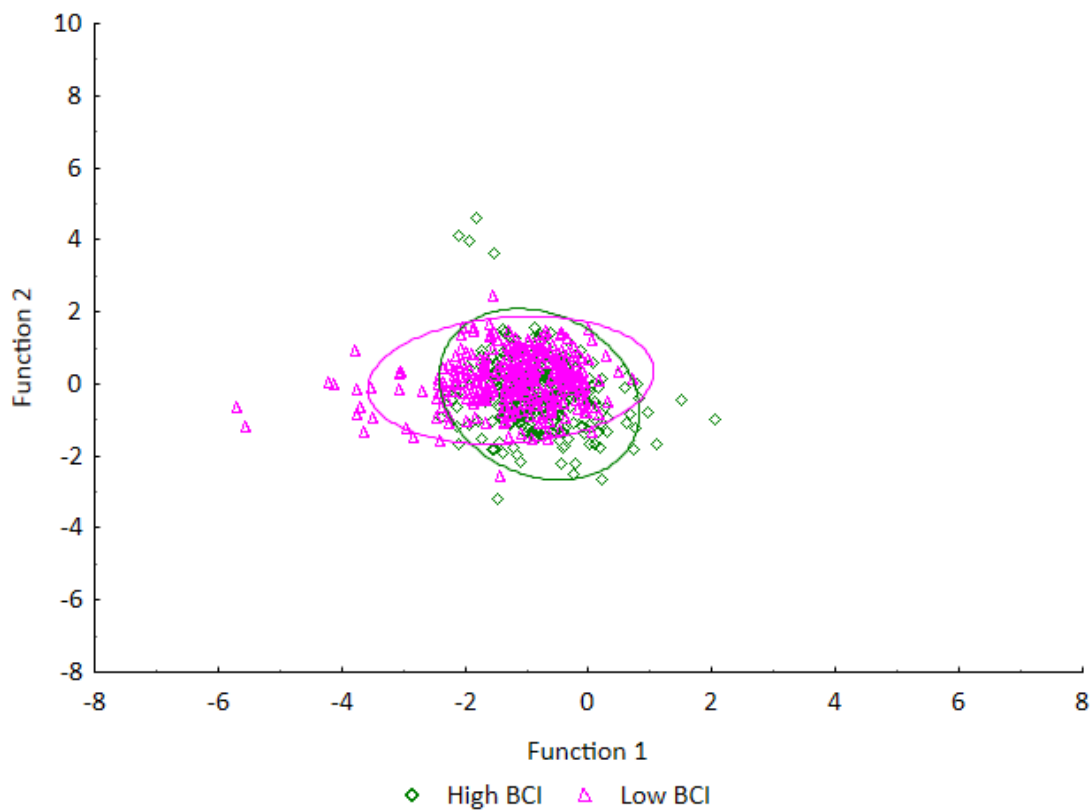
combinations differed significantly from one another (Table 4.3). The first discriminant function (Function 1) explained 94.6% of the variance and Function 2 explained a further 5.2% (Figure 4.3 and Figure 4.4). Call classification was low across all four phonic types (Male, Female, High BCI and Low BCI) on the basis of their acoustic parameters with an average classification success rate of <60%. Male classification success was lowest at 46.3%. Function 1 contained high loadings of PC1 and its discriminatory power is therefore mainly characterised by temporal parameters and the bandwidth of the initial FM component (D, IPI and BW<sub>i</sub>). Function 2 which had much lower discriminatory power was loaded heavily with PC3 and thus is characterised by spectral parameters (RF, F<sub>max</sub> and BW<sub>t</sub>).

**TABLE 4.3 F-VALUES OBTAINED FROM DISCRIMINANT FUNCTION ANALYSIS ON ECHOLOCATION CALL PARAMETERS OF FOUR PLAYBACK CATEGORIES (DF=4; ALL P<0.01).**

Species	Female	Male	High BCI	Low BCI
Female		39.6963	163.8414	236.393
Male	39.6964		65.5948	97.4323
High BCI	163.8414	65.5948		11.2519
Low BCI	236.3934	97.4323	11.2519	



**FIGURE 4.3** PLOT OF THE FIRST TWO FUNCTIONS OF A DFA ON TEN ACOUSTIC PARAMETERS OF THE MALE AND FEMALE PLAYBACK STIMULI. EACH CALL IS PLOTTED (N = 1192) AND COLOUR CODED FOR GENDER.



**FIGURE 4.4** PLOT OF THE FIRST TWO FUNCTIONS OF A DFA ON TEN ACOUSTIC PARAMETERS OF THE HIGH BCI AND LOW BCI PLAYBACK STIMULI. EACH CALL IS PLOTTED (N = 1192) AND COLOUR CODED FOR BCI.

The graphical plot of Functions 1 and 2 showed little separation between playback classes, with no discrete groupings visible, although they are statistically distinct. There is some separation between playback classes along Function 1 (call duration, interpulse interval and the bandwidth of the initial FM component) for males and females and along Function 2 for BCI. Calls of males and females (Figure 4.3) differed more (Mahalanobis distance = 1.13) than did the calls of high and low BCI individuals (Figure 4.4, Mahalanobis distance = 0.36).

**TABLE 4.4 SQUARED MAHALANOBIS DISTANCES OBTAINED FROM DISCRIMINANT FUNCTION ANALYSIS ON ECHOLOCATION CALL PARAMETERS OF FOUR PLAYBACK CATEGORIES.**

	<b>Female</b>	<b>Male</b>	<b>High BCI</b>	<b>Low BCI</b>
<b>Female</b>		1.128	2.107	4.044
<b>Male</b>	1.128		0.326	1.025
<b>High BCI</b>	2.107	0.326		0.359
<b>Low BCI</b>	4.044	1.025	0.359249	

#### 4.4 Discussion – Bats use multiple components of a signal for discrimination

This study provides experimental evidence that bats are able to use multiple components of echolocation calls to discriminate between the echolocation calls of other bats. *Rhinolophus clivosus* was able to successfully discriminate between synthesised echolocation calls which could be controlled to express changes in individual components of the calls. In addition to resting frequency, listening bats were able to detect changes in the FM components and in the duty cycle of calls. Much of the previous work on the subject focused on the frequency of the CF component in horseshoe bat calls as a marker to determine the role of echolocation in communication (Schuchmann and Siemers, 2010a; Li et al., 2014; Bastian and Jacobs, 2015), and in so doing revealed the potential for additional acoustic cues to influence discrimination when resting frequencies were similar.

The ability of bats to discriminate between calls of species with similar RF must be based on an ability to detect minute variations in additional call components, apart from peak frequency. Schuchmann and Siemers (2010a) originally suggested that the FM components of horseshoe bat calls show potential to encode such information. Bastian and Jacobs (2015) further verified the role of FM components and identified additional acoustic components as candidates for discrimination cues, namely call duration, duration to maximum intensity, minimum frequency of final FM component and call entropy. This study provides the first experimental evidence that bats can perceive differences in acoustic components separately from one another, supporting the use of multidimensional acoustic space in species discrimination. *Rhinolophus clivosus* was able to discriminate between calls that varied in RF, slope of the FM component, and duty cycle.

Of the three call components tested, changes in RF produced the highest discrimination success rate (Figure 4.1), suggesting that frequency provides the strongest recognition cue for species discrimination. However, there was no significant difference in the ability of bats to discriminate on the basis of frequency compared to either FM components or duty cycle. These other components therefore likely act as further discrimination cues. Species which differ significantly in call frequency may be immediately identified by a bat as distinct, but when frequencies are similar, these less prominent cues become important (Bastian and Jacobs, 2015). These results suggest that additional components of echolocation calls may still play a role in conveying species information, even when call frequencies are distinct. The importance of multiple acoustic components in discrimination is supported by the inability of *R. clivosus* to discriminate between  $Rca_{87}$  and  $Rbl_{87}$  (Chapter 3). In this case, the similarity between calls across all acoustic parameters inhibits *R. clivosus* from discriminating between

the two species. In contrast, in cases where call parameters differ even slightly in acoustic space, as in the case of  $Rcl_{92}$  and  $Rbl_{92}$  (Chapter 3, Figure 3.3), bats are able to discriminate.

The suggestion by Schuchmann and Siemers (2010a) and further analytical support by Bastian & Jacobs (2015) that FM components may facilitate discrimination is supported by the strong behavioural response by *R. clivosus* to changes in the parameters of the FM components of the playbacks in our experiments. The FM components of horseshoe bat echolocation calls, however, are complex in structure, and can vary in a number of ways. Due to limitations in the number of trials with which each bat could be presented, only a single manipulation of the FM component could be used in this study. In this case, bats were able to discriminate between calls with different sweep rates i.e. between calls with a long, narrow bandwidth FM component and calls with a short, broad bandwidth FM component. Even with just a single change in the slope of the FM component, bats showed a strong ability to discriminate between playbacks revealing sensitivity in the perception of this parameter. The possible range of variation, however, is far greater than that used here and provides opportunity for further investigation into the role of the FM component of horseshoe bat echolocation calls in communication. An aspect which deserves further attention is the difference between the initial and terminal FM components. Schuchmann and Siemers (2010a) as well as Bastian & Jacobs (2015) placed special emphasis on the potential importance of the terminal FM component, which is often more prominent. For this study, I manipulated both the initial and terminal FM components equally, although it is possible that a bat can vary these two components independently. Whether such manipulation by the sender, if it happens, facilitates or inhibits discrimination by the receiver remains to be discovered.

The communicative potential of the FM component may also shed additional light on the overall function and evolution of the FM component in HD-CF calls. The FM component in horseshoe bat echolocation calls is thought to function in determining target distance and location (Schnitzler, 1968; Simmons, 1973; Schnitzler and Denzinger, 2011). The spectral and temporal features of the FM component has been shown to vary depending on a number of external factors, including the level of ambient noise (Hage et al., 2014) as well as the presence of other bats, both con- and heterospecifics (Fawcett et al., 2015). The spectral features of the CF component are restricted by the acoustic fovea, limiting their ability to be adjusted to facilitate communication (but see (Jones and Ransome, 1993; Furusawa et al., 2012; Fawcett et al., 2015)). FM components on the other hand, as mentioned above, can be altered in response to a number of external factors.

Changes in the duty cycle of playback sequences also elicited a strong behavioural response from the listening bats. The duty cycle of echolocation calls has previously been shown to be of communicative value to bats (Gillam, 2007). During foraging, the pulse rate and duration of echolocation calls vary with each phase (search, approach and terminal) of an echolocation sequence (Gillam, 2007), resulting in a ‘feeding buzz’. Eavesdropping bats have been shown to be attracted to call sequences which include feeding buzzes, demonstrating the potential value of changes in duty cycle to a listening bat. This study, however, provides the first experimental evidence that a change in duty cycle alone, outside of the context of foraging can elicit a behavioural response from a listening bat.

The analysis of echolocation calls used in the species discrimination experiments (Chapter 3) suggested that when call frequencies were similar, as with Rcl<sub>92</sub> and Rbl<sub>92</sub>, temporal components explain the greatest level of interspecific variation. Duty cycle was therefore

likely a major contributing factor to the ability of *R. clivosus* to discriminate calls of *R. blasii* from those of conspecifics when frequencies overlapped. However, the Duty Cycle playback consisted of a combination of two call components, inter-pulse interval and call duration and it is not clear if it is the call duration or the inter-pulse interval that drives this response, or if the two components necessarily need to work in conjunction with one another and with other components. Future studies should investigate the separate effects of these components to gain a better understanding of which components are important to discrimination. The effect on communication of changes in duty cycle during flight should also be considered.

Although bats appear to be able to identify changes in individual components of echolocation, it is likely that all of these components are used in combination in discriminating amongst different individuals. The use of multiple components of the same signal in communication has been demonstrated in a number of animal groups including birds (Rivera-Gutierrez et al., 2010), frogs (Gerhardt, 1994) and humans (Murray and Arnott, 1993).

### **Can bats use echolocation to identify potential signals of reproductive fitness?**

Body condition can be used as an indicator of fitness and thus may play a role in mate choice and ultimately in the reproductive success of an individual. Therefore, an ability to identify body condition from echolocation calls could provide an honest signal for the female and therefore a reproductive advantage if fitter males produce more fertile sperm or fitter offspring. Only one study has provided evidence for echolocation-based mate choice in bats. Puechmaille *et al.* (Puechmaille et al., 2014), using call frequency as an indicator of body condition, demonstrated that female *R. mehelyi* preferentially selected males with higher echolocation frequencies which correlated with better body condition. Similarly, in my investigation, *R. clivosus* demonstrated a strong ability to discriminate between calls from

bats with high and low body condition, despite the calls of both body conditions occupying a similar acoustic space. However, analysis of the calls revealed that there were some differences, albeit small, amongst temporal and spectral characteristics including call duration, interpulse interval, resting frequency and the bandwidth of the FM components. The ability of *R. clivosus* to discriminate between echolocation calls from bats with differing body conditions suggests that *R. clivosus* has the potential to use echolocation in mate choice, as was shown for *R. mehelyi* (Puechmaille et al., 2014). The role of echolocation in mate choice of *R. clivosus* is further investigated in Chapter 5.

## Chapter 5

### **The Role of Echolocation in Individual Preference in Horseshoe bats.**

#### 5.1 Introduction

It is widely accepted that evolution and speciation rests largely on the basis of ecological divergence (West-Eberhard, 1983), with natural selection shaping the evolution of species as they adapt to variation in the environment. Not all diversion of traits, however, can be credited to natural selection. One notable exception is sexual selection. Charles Darwin originally described sexual selection as “an advantage which certain individuals have over other individuals of the same gender and species, in exclusive relation to reproduction” (Darwin, 1871).

Given a 1:1 population sex ratio, male-male competition for females can be high, and considering that the payoff for success is the ability to pass on their genes, selection for male ability to acquire mating partners can be very strong (Krebs and Davies, 1981). Sexual selection is thought to be driven by two primary mechanisms: male-male competition usually by sexual combat, and mate choice (Andersson and Iwasa, 1996; Panhuis et al., 2001; Paul, 2002). Sexual selection by female choice can drive the evolution of behavioural and morphological traits in males that attract and stimulate potential mates (Andersson and Iwasa, 1996). This can lead to the evolution of elaborate morphology and behaviours in males that are used in the courtship of females (Ryan, 1998). Female choice, in turn, is favoured if the preferred trait influences the immediate reproductive success of the female. More elaborate males, for example, may be better providers of resources or be of a lesser risk of transmitting venereal diseases or parasites (Ryan, 1998). The adaptation of traits through sexual

selection is driven by several mechanisms, including direct phenotypic effects, sensory bias, Fisherian runaway and 'good genes' or honest signalling indicator mechanisms (Andersson and Simmons, 2006). Direct phenotypic effects include male traits which directly reflect an individual's ability to provide material advantages such as parental care, protection or nutrition. Sensory bias occurs when males exploit a bias shown by females to traits which may have initially evolved under natural selection for other purposes. Fisherian runaway refers to exaggerated male ornaments, the evolution of which seem maladaptive and incompatible with natural selection. In this case, female selection for the trait may dilute the effects of natural selection on that trait. Indicator mechanisms are those that give an indication to females of the genetic quality of males carrying particular traits. Such genetic quality can, in turn, when selected by females, provide genetic benefits to their offspring (Andersson and Simmons, 2006).

Mate choice is influenced by multiple potential cues, often acting in conjunction with one another (Candolin, 2003). Such cues can take a variety of forms, including but not limited to visual displays, chemical and acoustic signals (McLean et al., 2013). Birds for instance may rely on a complex combination of brightly coloured ornamentation, courtship displays and elaborate song to select a mate (Candolin, 2003). Mate preference therefore relies on sensory and behavioural properties associated with a particular phenotype that can influence selection of a mate (Jennions and Petrie, 1997). Species recognition is also considered to be vital component of mate choice, with females necessarily needing to avoid mating with males from incompatible lineages which may lead to potentially dysfunctional hybrids (West-Eberhard, 1983). Many animal species, although seemingly indistinguishable from one another in appearance, maintain reproductive isolation via species specific cues which function in mate choice and sexual selection (Jones, 1997). For bat assemblages, the

convergence of phenotypic traits due to adaptations to similar habitats and foraging strategies leads to morphologically cryptic species which require such species specific cues to maintain reproductive isolation. Female preference therefore acts as a major selective force on male signals (Ptacek, 2000; McLean et al., 2013). This selection can drive rapid divergence in a trait in evolutionary timeframes (Panhuis et al., 2001; McLean et al., 2013) and ultimately lead to greater reproductive isolation (Panhuis et al., 2001).

Acoustic signals commonly function in mate choice in a number of animals (Searcy and Andersson, 1986), most notably in anurans (Gerhardt, 1992; Gerhardt, 1994; Amezcuita et al., 2006; McLean et al., 2013) and birds (Slabbekoorn and Smith, 2002; Byers and Kroodsma, 2009; Riebel, 2009; Price, 2013). The most commonly heard frog calls are what is referred to as advertisement calls, used to attract females, but which function not only to attract females, but also in aggressive interactions with other males (Gerhardt, 1994). Males exert large amounts of energy producing advertisement calls, which are then used by females to select their mates, as these calls not only announce their presence, but also transmit information about the size and quality of the individual (McLean et al., 2013). Bird song is another acoustic signal that plays a major role in both intrasexual competition for mates and resources, but also as an important signal for female choice (Riebel, 2009). Competition for mates has led to the evolution of complex repertoires of song with great variation between species (Riebel, 2009), with females often showing preference for males with more extensive song repertoires (Byers and Kroodsma, 2009; Price, 2013).

For nocturnal animals such as bats, which spend much of their lives in the dark, visual cues become limited, promoting the use of non-visual cues for mate choice, such as olfactory and acoustic signals (Voigt et al., 2008). Bat communication therefore relies heavily on acoustic

signals (Jones, 1997). Whereas bat social calls are known to function in mate choice in bats (Behr and von Helversen, 2004; Andrews et al., 2006; Sachteleben and von Helversen, 2006), there is another widely available acoustic signal that may hold potential cues to serve in mate choice - echolocation.

With the communicative potential of bat echolocation calls to transmit information on individual identity (Masters et al., 1995; Fenton et al., 2004; Carter et al., 2008; Yovel et al., 2009), gender (Masters et al., 1995; Kazial and Masters, 2004; Knörnschild et al., 2012; Finger, 2015), species identity (Jones and Van Parijs, 1993; Jones, 1997; Kingston et al., 2001; Mayer and von Helversen, 2001; Schuchmann and Siemers, 2010a; Li et al., 2014; Bastian and Jacobs, 2015) and colony membership (Pearl and Fenton, 1996; Boughman and Wilkinson, 1998; Esser and Schubert, 1998; Voigt-Heucke et al., 2010), there is a possibility of bats using echolocation calls to select mates. Echolocation calls, however, which function in orientation and foraging, are strongly shaped by natural selection to exploit the ecological niche in which a bat forages (Neuweiler, 1989). This places major constraints on the variability in echolocation calls which may facilitate mate preference. Similar constraints have been found to have a strong influence on the evolution of anuran vocalisations (Gerhardt, 1994; McLean et al., 2013) and that sexual selection on call design is not enough to overshadow the phylogenetic constraints (McLean et al., 2013). In the case of bat echolocation, species and individual specific signals must therefore be contained in small intra- and interspecific differences in echolocation calls to act as communicative cues, maintaining the structure and function of the calls required to facilitate their function in orientation and foraging (Bastian and Jacobs, 2015).

Although a number of studies have suggested a potential role of echolocation in the context of sexual selection (Jones, 1997; Jones and Siemers, 2010; Knörnschild et al., 2012), only two studies have looked directly at the role of echolocation in mate choice in bats. The first investigation into the direct role of echolocation calls in the sexual selection of bats looked at the preference for varied call frequencies in *R. mehelyi* (Puechmaille et al., 2014). Initially, they identified a significant correlation between body condition and peak echolocation frequency. Individuals with higher body condition scores also exhibited higher peak echolocation frequencies. Call frequency was then used as a proxy for body condition in a two-alternative choice experiment. Test individuals were subjected to two playbacks simultaneously, one consisting of high frequency echolocation calls and the other using low frequency echolocation calls, and the level of female preference was recorded for each stimulus. Results found that during the mating season, female *R. mehelyi* select males with high frequency echolocation calls. The impact of this in terms of sexual selection was supported by genetic analysis of relatedness, finding that high frequency males sire more offspring.

Grilliot et al. (2015) have since investigated preference of the big brown bat, *Eptesicus fuscus*, for echolocation calls from individuals of the opposite gender with varying levels of copulatory frequency. They found that males showed preference for echolocation calls from females with high copulatory frequency, but found no evidence for preference in females. This suggests at least some level of male-choice sexual selection in big brown bats. Although female-choice is considered the primary driver of sexual selection due to the high costs of reproduction to females (Andersson and Simmons, 2006), some studies have suggested a potential cost of producing ejaculate to males, resulting in male-choice influencing sexual selection (Dewsbury, 1982)

A necessary first step for uncovering the link between mate choice and echolocation is to determine if bats can show a preference based on echolocation. I investigated this in *R. clivosus* using an experimental playback approach to test for preference for both gender and body condition

H<sub>1</sub>: *R. clivosus* show preference for echolocation calls produced by individuals of the opposite gender with good body condition.

H<sub>0</sub>: *R. clivosus* shows no preference for echolocation calls produced by individuals with good body condition.

Based on the findings of Puechmaille et al. (2014), one would predict that *R. clivosus* would show preference for calls from individuals of the opposite gender and with higher body condition.

## 5.2 Methods for Preference Trials

### 5.2.1 Study Period

To achieve an accurate investigation of mate choice, the study must be done during the mating season when test individuals are sexually active. The timing of this study, therefore, was based on the findings of two previous studies which investigated the timing of mating in two regions further north in South Africa. These studies, however, show two separate mating seasons, dependent on the location of the population. In KwaZulu-Natal, a coastal region with a warm, subtropical climate, copulation is observed in May, before the onset of winter (Bernard, 1983). Sperm is then stored in the oviducts and uterine horns of the females through winter, with fertilization occurring in August. In Gauteng, an interior region with a mild climate, however, sperm is not uniformly stored through winter by females, suggesting

that sperm storage occurs in males (Wessels and van der Merwe, 1997). Evidence of copulation was therefore observed throughout the winter months, peaking in early spring. De Hoop Nature Reserve, the site of this study, is situated in a Mediterranean climate, with warm summers and mild winters, making it difficult to accurately match the mating season with either of the previous studies. This study therefore covered both periods, repeating the same set of experiments in autumn and spring.

### 5.2.2 Preparation of Playback Files

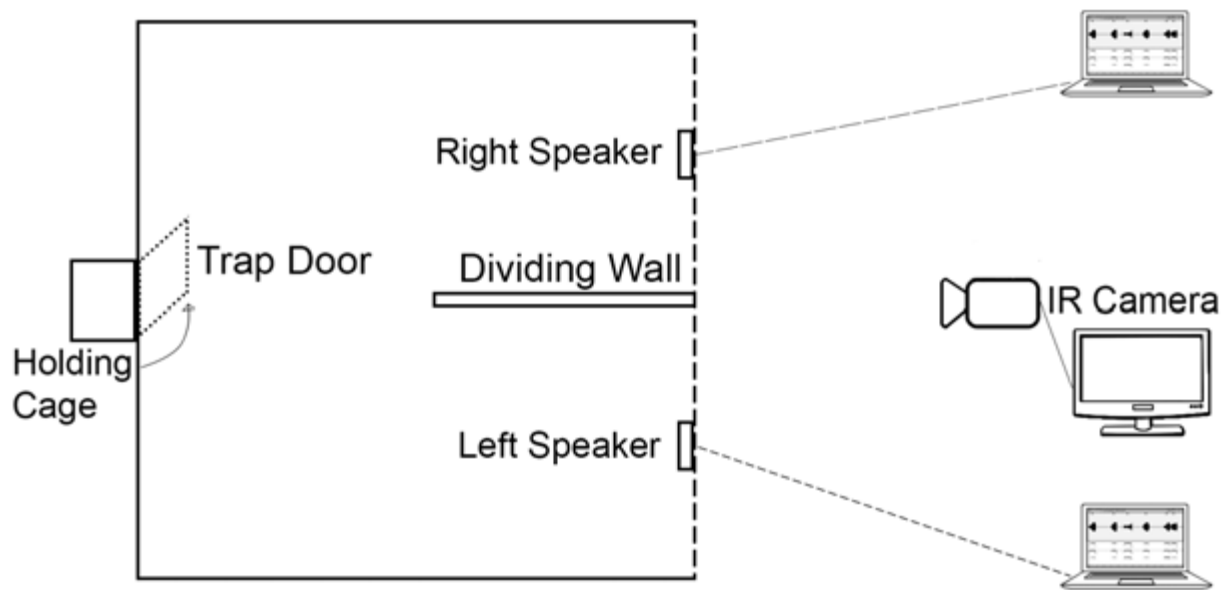
Semi-synthetic duplicate calls of *R. clivosus* (see section 2.4) were divided into four categories: Female, Male, High BCI, Low BCI. At least 200 echolocation calls were selected from each category, compiled from multiple individuals (Females: N = 20, Males: N = 20, High BCI: N = 5, Low BCI N=5). For the High BCI and Low BCI categories, calls from the five male individuals with the highest and lowest BCI values respectively were selected from an existing database of the UCT Animal Evolution and Systematics group.

The semi-synthetic calls were then compiled randomly into individual playback files (duration = 240 s) for each of the four categories. Inter-pulse intervals were selected for each call individually, based on the inter-pulse interval preceding the original call. Call intensities were normalised for intensity using automated normalisation (Avisoft-SASLab Pro, v.5.2.07, Avisoft Bioacoustics, Glienicke, Germany) within and across all categories. The playback files were then paired into three playback combinations: 1. Control (Male-Male), Gender (Male-Female) and BCI (High BCI-Low BCI).

### 5.2.3 Experimental procedures

Two-alternative choice experiments were conducted on wild caught adult *R. clivosus* individuals. Experiments took place in a wooden experimental chamber (Figure 5.1). The

chamber was lined on all external surfaces with sound absorbing foam to limit the influence of external factors on the experiments, with the exception of one end, which was covered only with plastic mesh, allowing camera surveillance within the chamber. The open end was divided down the middle, extending to the midpoint of the box, creating two defined compartments. The mesh provided the only substantial perch for bats to land on within the experimental chamber, encouraging bats to make a decision. An ultrasound speaker (Avisoft UltraSoundGate Player BL Light, Avisoft Bioacoustics, Glienicke, Germany) was placed at the top centre of each compartment and connected to a laptop (Asus Eee PC Seashell Series). A trapdoor (200mm x 300mm) was situated three quarters of the way up the opposing end, centred with the divider, with a small external holding cage placed on the outside. The trapdoor could be opened from outside the box via a small rope. Behavioural responses were recorded using an infrared video camera (Bell + Howell DNV16HDZ, New York, USA), placed 2m outside the open end of the experimental box. Acoustic responses were recorded using an ultrasound microphone (Avisoft UltraSoundGate 416, Avisoft Bioacoustics, Glienicke, Germany) and Avisoft-Recorder software (Version 4.2, Avisoft Bioacoustics, Glienicke, Germany) on a Lenovo Ideapad laptop (Model Y580).



**FIGURE 5.1 EXPERIMENTAL SETUP IN THE SOUND ATTENUATING CHAMBER VIEWED FROM THE TOP (WOODEN BOX, LINED ON EXTERNAL SURFACES WITH SOUND ABSORBING FOAM (SOLID LINES) OR PLASTIC MESH (DASHED LINE)). INTERNAL DIMENSIONS: 125 X 125 X 125 CM. TOP-DOWN VIEW), FITTED WITH AN TWO ULTRASOUND SPEAKERS, ONE ON EITHER SIDE OF A DIVIDING WALL BROADCASTING THE PLAYBACK STIMULI SIMULTANEOUSLY. THE BOX WAS MONITORED BY AN INFRARED VIDEO CAMERA PLACED APPROXIMATELY 2 M FROM THE OPEN END AND CONNECTED TO AN EXTERNAL MONITOR. A SMALL HOLDING CAGE WITH TRAP DOOR WAS POSITIONED ON THE OPPOSING WALL TO THE SPEAKERS, FROM WHICH THE BAT WAS RELEASED.**

Bats were placed inside the external holding cage and allowed time to settle in silence. Once the bat was settled, defined by lack of movement of the body, the stimuli were presented. The trapdoor was opened following a three second delay to allow the bats time to register the signals before release. The bats reactions and movements within the box were recorded for 60s after the opening of the trapdoor.

Each experimental trial contained a single playback combination (e.g. gender), with one stimulus category presented through each speaker (e.g. male or females calls). Each individual was presented with all three combinations in a single night. Bats were placed in cotton bat bags in a quiet room and allowed at least 45 minutes to settle between trials. The order in which playback combinations were presented was randomised both among and within individuals to avoid any effect of trial order on behavioural responses to any particular

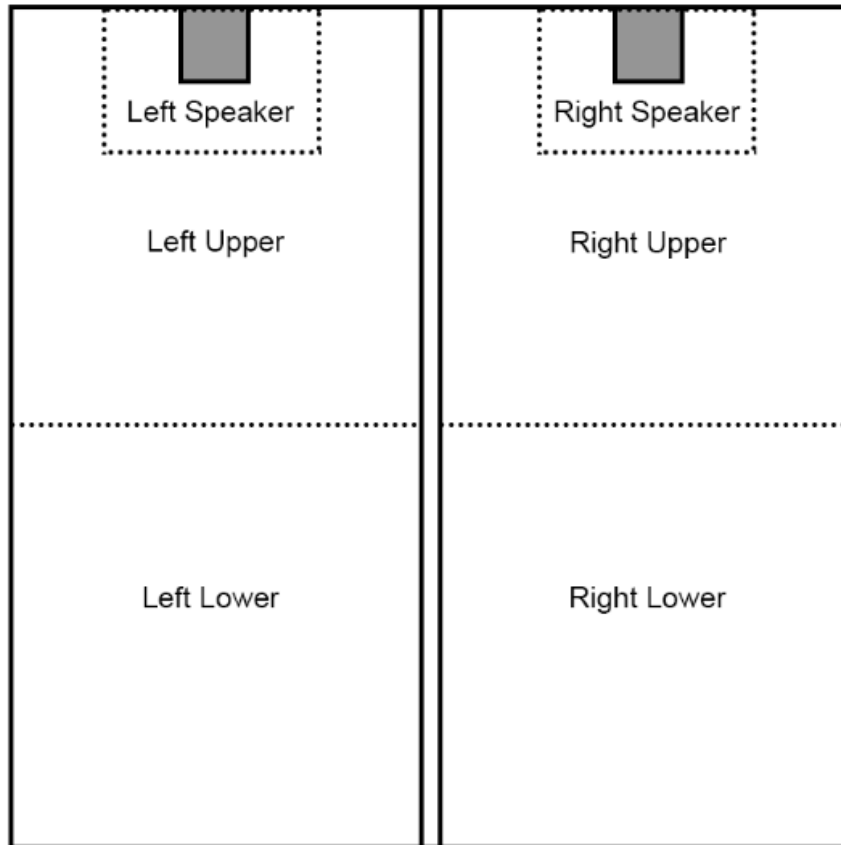
playback combination. The side from which each playback was presented was randomised within each combination, to ensure that any preference observed was to the stimulus and not to any external factors influencing particular sides of the experimental setup.

The experiment was duplicated across two seasons to correspond with potential seasonality of mating (Bernard, 1983; Wessels and van der Merwe, 1997). Austral Spring trials were conducted in September 2014 and Austral Autumn trials were conducted in April-May 2015.

#### 5.2.4 Behavioural Response – Video Analyses

The bats' behavioural response, in particular their movements within the experimental box were measured from video recordings for 60 s from the point at which the trapdoor was fully open. Only trials in which the bat emerged from the holding cage were included in the analyses.

The open end of the box was divided into three end zone sections for each side (left and right) of the divide, referred to here as Speaker, Upper and Lower sections giving a total of six sections (Figure 5.2). The Solomon Coder (Version 14.05.18, András Péter, Hungary, [www.solomoncoder.com](http://www.solomoncoder.com)) was used to record the duration of time the bats were perched within each of the six sections. The time delay for bats exiting the holding cage (measured from the point at which the trapdoor was fully open to the point at which the bat first took flight) was recorded, together with direction of first flight and the side on which the bat first settled. The duration of time in flight on either side and the number of times the bat switched from one side to the other was also recorded.



**FIGURE 5.2** END ZONE SECTIONS SET ACROSS THE OPEN END OF THE EXPERIMENTAL BOX, VIEWED FROM THE FRONT. EACH SIDE OF THE DIVIDING WALL WAS SUBDIVIDED DOWN THE MIDDLE TO CREATE AN UPPER AND LOWER HALF. THE UPPER HALF WAS FURTHER SUBDIVIDED TO FORM A SPEAKER SECTION (A 10 CM PERIMETER IMMEDIATELY SURROUNDING EACH SPEAKER) AND AN UPPER SECTION (THE AREA ABOVE THE MIDDLE LINE, EXCLUDING THE SPEAKER SECTION). EACH LOWER, UPPER AND SPEAKER SECTIONS COMPRISED OF 25%, 20% AND 5% OF THE TOTAL END ZONE AREA RESPECTIVELY.

### 5.2.5 Statistics

All statistical testing, with the exception of the preference model, was conducted using Statistica 64 (Version 11, StatSoft Inc., Tulsa, USA). Preference models were calculated manually using Microsoft Excel. A significance level of  $\alpha=0.05$  was used for all analyses. Shapiro-Wilks tests were used to test for normality in the data. Data were compared between seasons to test if the relative durations spent on either side of the divide differed between seasons using Mann-Whitney U Test. To test if the side of the experimental box that a signal was played from had an effect on the outcome of the test, the durations of time bats spent on each side of the divide (regardless of stimuli) was compared for all trials combined using

Wilcoxon Pairs Tests. For the above mentioned tests, data from all three end zone sections (Speaker, Upper, Lower) were combined for each side separately. To test if there was a difference in bat reaction time between playback combinations, the time delay from the opening of the trap door to the point at which the bats first exited the holding cage was compared between playback combinations using a Kruskal-Wallis ANOVA. Multiple regressions were used to investigate the relationship between body condition and echolocation parameters.

A habitat preference model was modified to test if preference was shown to any particular section of the experimental box. The model compares observed durations within each section to their availability (percentage of each particular section in relation to the overall available space which accounts for differences in size between the end zone sections) to determine selection ratios for each section (Manly et al., 1993). Preference scores were calculated using the following equation:

$$\hat{w}_i = \frac{o_i}{\pi_i}$$

Where  $o_i$  is the proportion of time spent in section  $i$ ,  $\pi_i$  is the proportional availability of section  $i$ , and  $\hat{w}_i$  is the preference score for section  $i$ .

Standard error of  $\hat{w}_i$  was calculated by

$$SE(\hat{w}_i) = \hat{w}_i \sqrt{\frac{1}{u_i} - \frac{1}{u_+} + \frac{1}{m_i} - \frac{1}{m_+}}$$

Where  $u_i$  is the total duration of time spent in section  $i$ ,  $u_+$  is the total time spent across all sections,  $m_i$  is the relative availability of section  $i$ , and  $m_+$  is the total available space.

$w_i$  95% confidence intervals (CI) calculated by

$$w_i \pm Z_{\alpha/2I} SE_{(w_i)}$$

Where  $Z_{(\alpha/2I)}$  is the  $100\alpha/(2I)$  percentage point of the standard normal distribution and  $I$  is the number of sections. Bonferroni correction applied by dividing the  $\alpha$  significance level (0.05) by the number of sections to allow for multiple comparisons.

Confidence intervals were used to determine the significance of preference scores. Upper confidence values of  $<1$  suggest avoidance for that particular section. Lower confidence values of  $>1$  suggest significant preference for that particular section. All confidence values outside of these bounds suggest that that particular section is used in proportion to its availability.

For each playback combination (Control, Gender and BCI), potential preference for one playback stimulus over the other was tested on three levels. Firstly, the total number of times the bats landed on the side of each stimulus (all end zone sections combined) was compared using a Fisher's Exact test. This was then further analysed to include the durations perched on each side using Wilcoxon Pairs tests. Finally, the durations were refined to only include the durations spent within the speaker section of the end zone.

### 5.3 Results for Preference Trials.

A total of 70 *R. clivosus* individuals were tested, comprising 45 in Spring (Males:  $N = 22$ ; Females:  $N = 23$ ) and 25 in Autumn (Males:  $N = 12$ ; Females:  $N = 13$ ). The durations spent on either side of the box were found to be non-normally distributed for all three experimental trials across both seasons (Shapiro-Wilk: Spring (Trial A),  $W = 0.54$ ,  $p < 0.001$ ; Spring (Trial B),  $W = 0.45$ ,  $p < 0.001$ ; Spring (Trial C),  $W = 0.49$ ,  $p < 0.001$ ; Autumn (Trial A),  $W = 0.87$ ,  $p < 0.001$ ;

Autumn (Trial B),  $W = 0.61$ ,  $p < 0.001$ ; Autumn (Trial C),  $W = 0.58$ ,  $p < 0.001$ ), therefore non-parametric tests were used for subsequent analysis. The duration spent on either side was not significantly different between the two seasons for either gender (Mann-Whitney U Test: Male:  $U = 6$ ,  $Z = -0.53$ ,  $p > 0.05$ ; Female,  $U = 18$ ,  $Z = -0.91$ ,  $p > 0.05$ ), thus seasonal data were pooled for the remainder of the analysis. The side of the box from which each playback was presented was found to have no influence on the outcome of tests combinations (Wilcoxon Pairs Test  $Z = 0.86$ ,  $p > 0.05$ ), which together with the randomisation of playbacks eliminates the potential influence of external factors influencing preference for one side of the box. Any preference observed for one playback over another can therefore be considered preference to the acoustic stimuli and not to external factors. There was no significant difference in the time delay for bats exiting the holding cage between playback categories for both males and females (Kruskal-Wallis: Males:  $H(2) = 5.1$ ,  $p > 0.05$ ; Females:  $H(2) = 0.47$ ,  $p > 0.05$ ). Bats were therefore equally attracted to leave the holding cage and explore the experimental box for all trials, regardless of the playbacks presented.

Significant preference was shown to the speaker sections on both sides of the experimental chamber, for all experimental trials and across both genders (Table 5.1). In contrast, almost all other sections were avoided relative to the area they provided (Table 5.1). The strong preference shown by the bats to the speakers suggests that the bats are being attracted to the playbacks. An alternative explanation could be that the speakers are placed in the upper region of the enclosure and the bats are being attracted to the height as opposed to speaker itself, but the avoidance of other upper sections suggests that the speaker is likely attracting the bats. Results were therefore refined to not only look at each side as a whole, but to focus on the area surrounding the speaker in particular.

There was no significant difference in the number of times the bats landed on either side of the experimental box for males (Figure 5.3) and females (Figure 5.4) across all three playback combinations (Fishers Exact: Trial A (Male):  $P > 0.05$ ; Trial A (Female):  $P > 0.05$ ; Trial B (Male):  $P > 0.05$ ; Trial B (Female):  $P > 0.05$ ; Trial C (Male):  $P > 0.05$ ; Trial C (Female):  $P > 0.05$ ).

**TABLE 5.1 PREFERENCE SCORES FOR EACH OF THE SECTION OF THE EXPERIMENTAL CHAMBER ACROSS BOTH GENDERS AND ALL THREE TRIALS. AREAS OF SIGNIFICANT AVOIDANCE ARE DISPLAYED IN BOLD IN THE CI UPPER COLUMN AND AREAS OF SIGNIFICANT PREFERENCE ARE DISPLAYED IN BOLD IN THE CI LOWER COLUMN.**

Gender	Trial	Section	Used	$\alpha$ Used	$\alpha$ Available	$\hat{w}_i$	SE	CI Upper	CI Lower
Female	Control	Left Speaker	73.10	0.19	0.05	3.86	1.73	4.59	<b>3.14</b>
		Left Upper	52.42	0.14	0.20	0.69	0.16	<b>0.76</b>	0.62
		Left Lower	17.06	0.05	0.25	0.18	0.05	<b>0.20</b>	0.16
		Right Speaker	131.90	0.35	0.05	6.97	3.08	8.26	<b>5.69</b>
		Right Upper	101.18	0.27	0.20	1.34	0.29	1.46	<b>1.22</b>
		Right Lower	2.62	0.01	0.25	0.03	0.02	<b>0.04</b>	0.02
	Gender	Left Speaker	121.14	0.41	0.05	8.29	3.66	9.81	<b>6.76</b>
		Left Upper	25.40	0.09	0.20	0.43	0.12	<b>0.48</b>	0.38
		Left Lower	9.46	0.03	0.25	0.13	0.05	<b>0.15</b>	0.11
		Right Speaker	79.72	0.27	0.05	5.45	2.43	6.47	<b>4.44</b>
		Right Upper	31.08	0.11	0.20	0.53	0.14	<b>0.59</b>	0.47
		Right Lower	25.58	0.09	0.25	0.35	0.09	<b>0.39</b>	0.31
	BCI	Left Speaker	67.98	0.32	0.05	6.49	2.90	7.70	<b>5.28</b>
		Left Upper	29.84	0.14	0.20	0.71	0.19	<b>0.79</b>	0.63
		Left Lower	8.54	0.04	0.25	0.16	0.06	<b>0.19</b>	0.14
		Right Speaker	54.34	0.26	0.05	5.19	2.34	6.16	<b>4.21</b>
		Right Upper	6.28	0.03	0.20	0.15	0.07	<b>0.18</b>	0.12
		Right Lower	42.60	0.20	0.25	0.81	0.18	<b>0.89</b>	0.74
Male	Control	Left Speaker	115.90	0.38	0.05	7.61	3.37	9.02	<b>6.21</b>
		Left Upper	36.76	0.12	0.20	0.60	0.15	<b>0.67</b>	0.54
		Left Lower	16.74	0.05	0.25	0.22	0.06	<b>0.25</b>	0.19
		Right Speaker	110.98	0.36	0.05	7.29	3.23	8.64	<b>5.95</b>
		Right Upper	13.94	0.05	0.20	0.23	0.08	<b>0.26</b>	0.20
		Right Lower	10.10	0.03	0.25	0.13	0.05	<b>0.15</b>	0.11
	Gender	Left Speaker	137.76	0.57	0.05	11.34	4.98	13.41	<b>9.26</b>
		Left Upper	0.00	0.00	0.20	0.00	0.00	<b>0.00</b>	0.00
		Left Lower	36.24	0.15	0.25	0.60	0.14	<b>0.65</b>	0.54
		Right Speaker	50.46	0.21	0.05	4.15	1.88	4.94	<b>3.37</b>
		Right Upper	18.56	0.08	0.20	0.38	0.11	<b>0.43</b>	0.33
		Right Lower	0.00	0.00	0.25	0.00	0.00	<b>0.00</b>	0.00
	BCI	Left Speaker	66.94	0.22	0.05	4.35	1.96	5.17	<b>3.54</b>
		Left Upper	67.06	0.22	0.20	1.09	0.25	1.19	0.99
		Left Lower	0.00	0.00	0.25	0.00	0.00	<b>0.00</b>	0.00
		Right Speaker	161.70	0.53	0.05	10.52	4.62	12.44	<b>8.59</b>
		Right Upper	11.76	0.04	0.20	0.19	0.07	<b>0.22</b>	0.16
		Right Lower	0.00	0.00	0.25	0.00	0.00	<b>0.00</b>	0.00

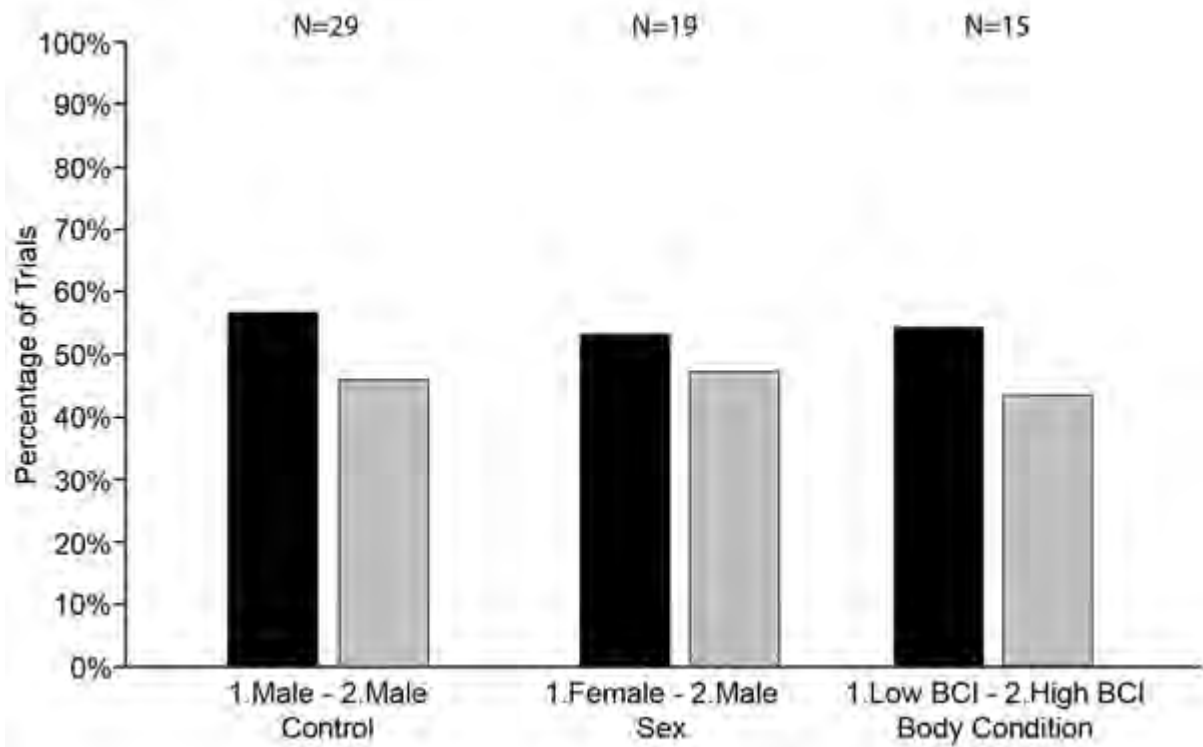


FIGURE 5.3 PERCENTAGE OF LANDINGS FOR EACH STIMULUS BY *RHINOLOPHUS CLIVOSUS* MALES. BLACK INDICATES THE PERCENTAGE OF LANDINGS AT STIMULUS 1. GREY INDICATES THE PERCENTAGE OF LANDINGS AT STIMULUS 2.

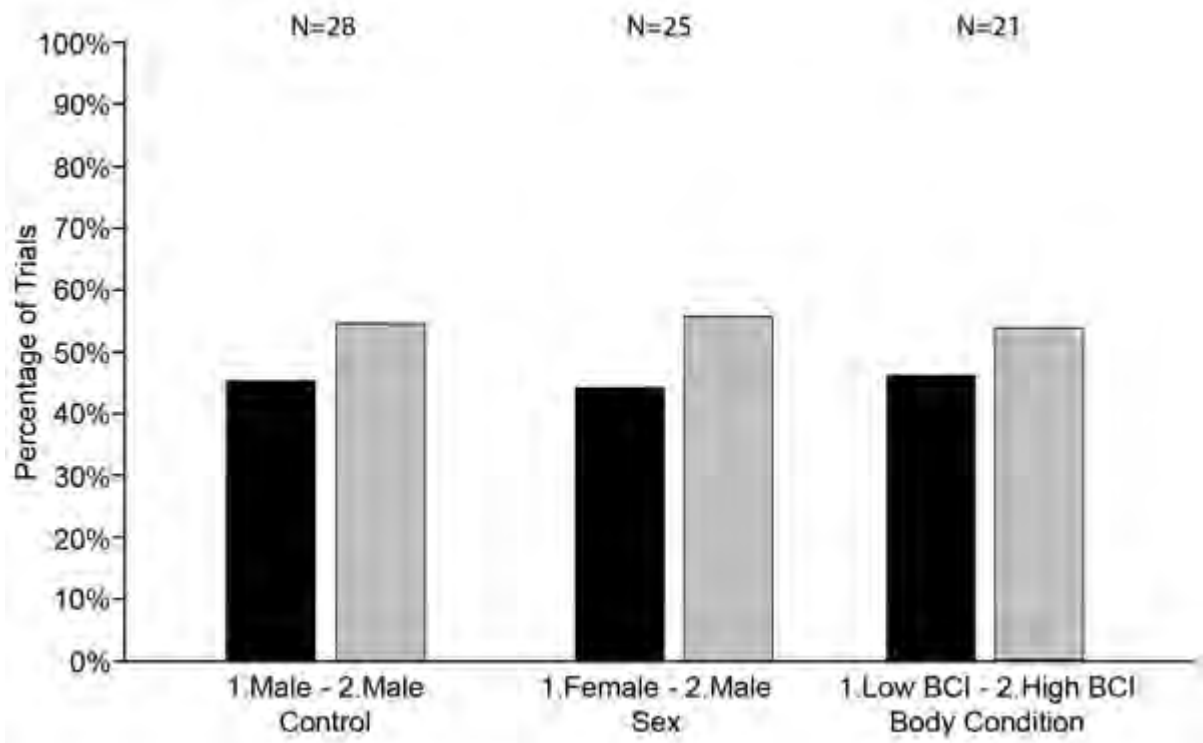
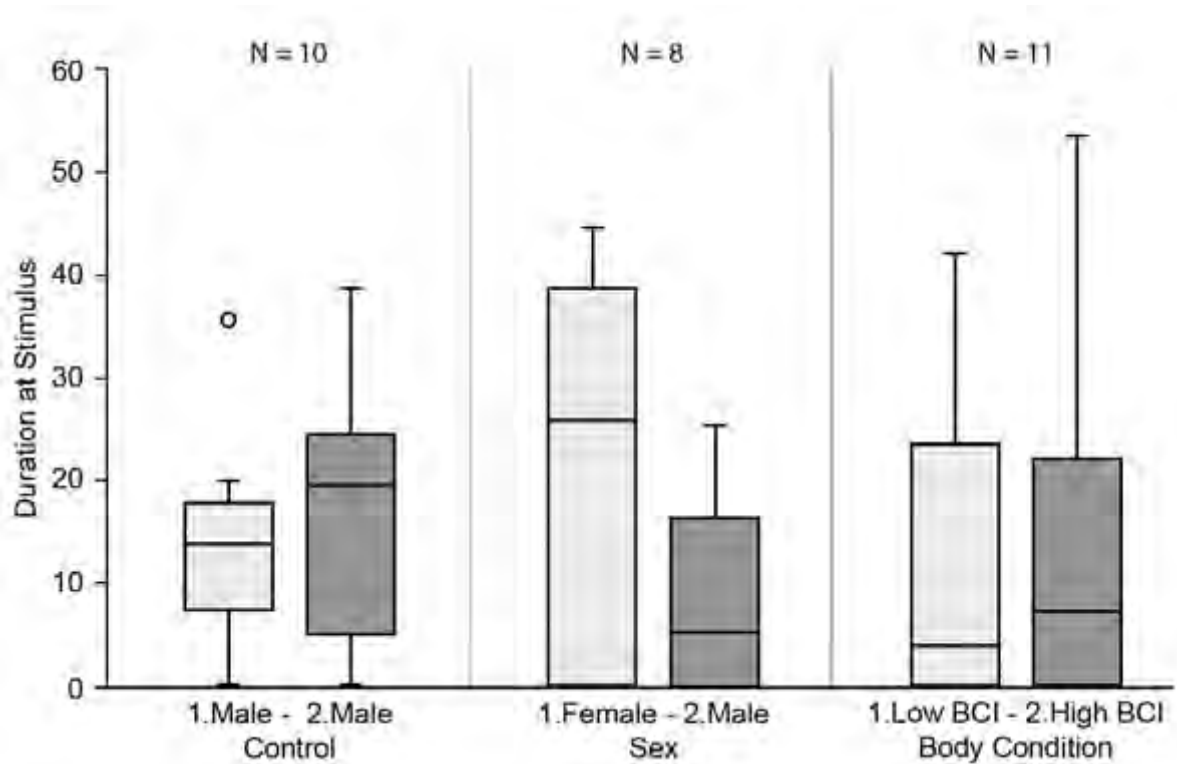


FIGURE 5.4 PERCENTAGE OF LANDINGS ON EITHER SIDE OF THE BOX BY *RHINOLOPHUS CLIVOSUS* FEMALES. BLACK INDICATES THE PERCENTAGE OF LANDINGS AT STIMULUS 1. GREY INDICATES THE PERCENTAGE OF LANDINGS AT STIMULUS 2.

The lack of preference observed in the number of times a bat landed at each stimulus is further supported in the duration spent perched in the vicinity of the stimulus. No significant difference was observed between the amount of time spent at each stimulus for either gender across any of the three categories (Wilcoxon Pairs Test: Trial A (Male):  $Z = 0.76, p > 0.05$ ; Trial A (Female):  $Z = 0.91, p > 0.05$ ; Trial B (Male):  $Z = 1.35, p > 0.05$ ; Trial B (Female):  $Z = 0.18, p > 0.05$ ; Trial C (Male):  $Z = 0.27, p > 0.05$ ; Trial C (Female):  $Z = 0.05, p > 0.05$ ; Figure 5.5, Figure 5.6). The same result was observed when data were refined to only include the time spent landed in the speaker sections (Wilcoxon Pairs Test: Trial A (Male):  $Z = 0.30, p > 0.05$ ; Trial A (Female):  $Z = 0.70, p > 0.05$ ; Trial B (Male):  $Z = 1.15, p > 0.05$ ; Trial B (Female):  $Z = 0.53, p > 0.05$ ; Trial C (Male):  $Z = 0.97, p > 0.05$ ; Trial C (Female):  $Z = 0.42, p > 0.05$ ).



**FIGURE 5.5 DURATIONS SPENT BY MALE *R. CLIVOSUS* AT EACH STIMULUS, WITH BOXES REPRESENTING INTERQUARTILE RANGE AND LINES REPRESENTING THE MEDIAN. WHISKERS SHOW NON-OUTLIER RANGE WITHIN 1.5 TIMES THE INTERQUARTILE RANGE. OUTLIERS OUTSIDE OF 1.5 TIMES THE INTERQUARTILE RANGE ARE PRESENTED WITH CIRCLES.**

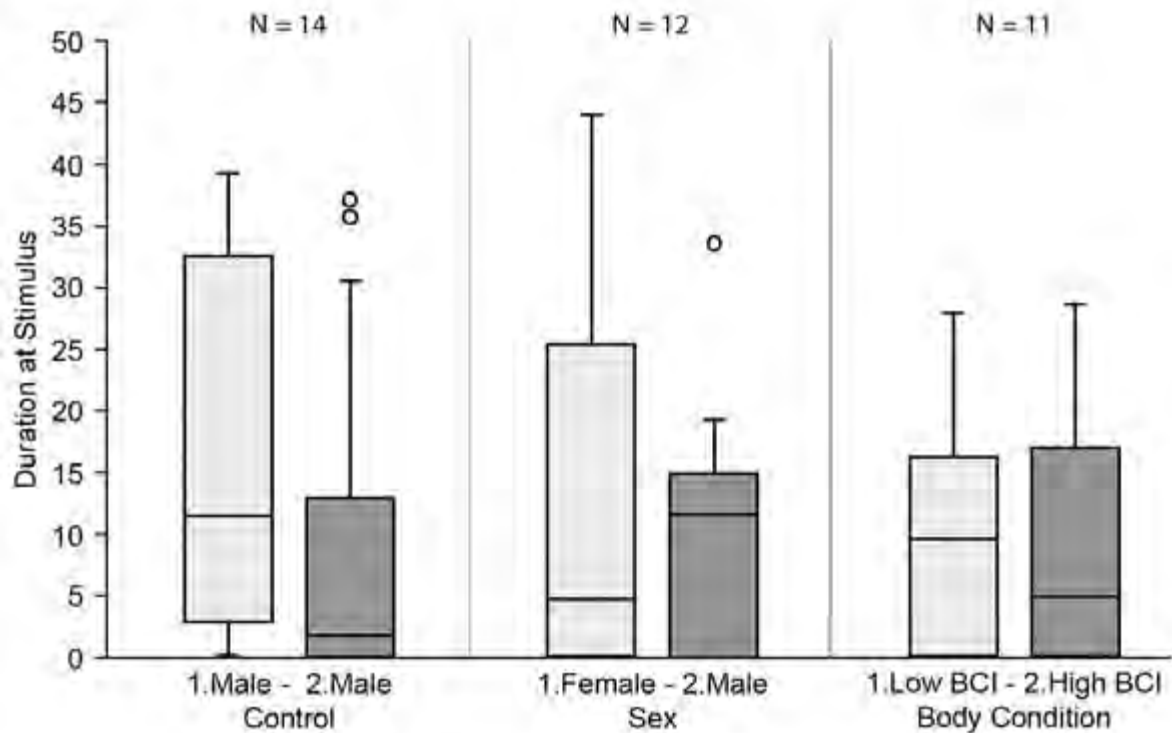
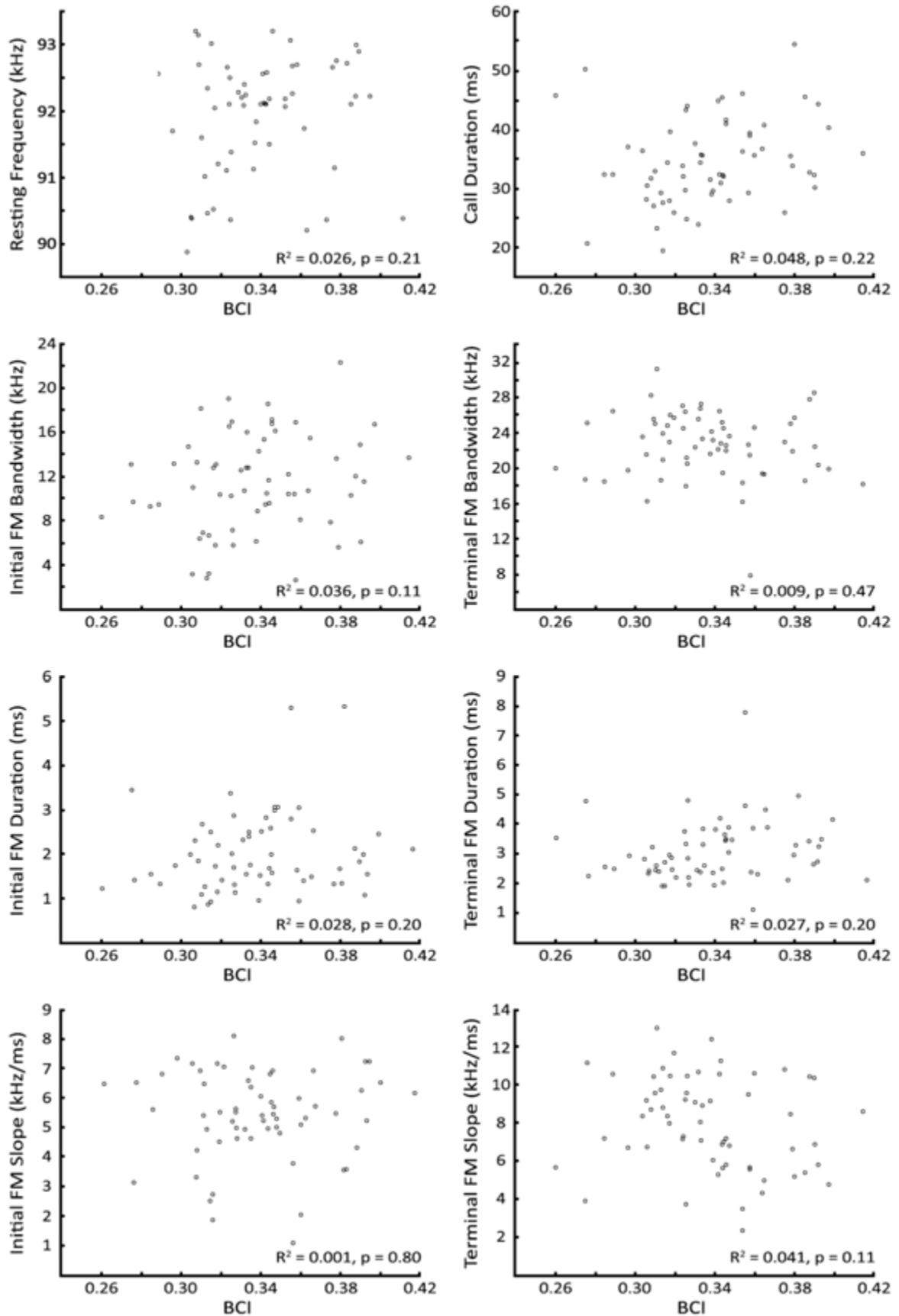


FIGURE 5.6 DURATIONS SPENT BY FEMALE *R. CLIVOSUS* AT EACH STIMULUS, WITH BOXES REPRESENTING INTERQUARTILE RANGE AND LINES REPRESENTING THE MEDIAN. WHISKERS SHOW NON-OUTLIER RANGE WITHIN 1.5 TIMES THE INTERQUARTILE RANGE. OUTLIERS OUTSIDE OF 1.5 TIMES THE INTERQUARTILE RANGE ARE PRESENTED WITH CIRCLES.

No relationship was found between BCI and any of the echolocation call parameters measured for the analysis (Multiple Regression:  $R = 0.29$ ,  $df = 8$ ,  $F = 0.63$ ,  $p > 0.05$ , Table 5.2, Figure 5.7). This suggests that there is no single component in the echolocation calls of *R. clivosus* that can be used to predict body condition.

TABLE 5.2 MULTIPLE REGRESSION EFFECT SIZES OF EIGHT ECHOLOCATION PARAMETERS ON BCI. DESCRIPTIONS OF CALL PARAMETERS ARE LISTED IN TABLE 3.1.

	SS	Degrees of Freedom	MS	F	P
Intercept	0.0002	1	0.0002	0.1685	0.6831
RF	0.0013	1	0.0013	1.1858	0.2811
D	0.0002	1	0.0002	0.1870	0.6672
BW <sub>i</sub>	0.0004	1	0.0004	0.3659	0.5478
D <sub>i</sub>	0.0002	1	0.0002	0.1669	0.6845
Sr <sub>i</sub>	0.0001	1	0.0001	0.0977	0.7558
BW <sub>t</sub>	0.0005	1	0.0005	0.4993	0.4829
D <sub>t</sub>	0.0002	1	0.0002	0.1994	0.6570
SR <sub>t</sub>	0.0001	1	0.0001	0.1211	0.7293



**FIGURE 5.7 RELATIONSHIP BETWEEN BCI AND EIGHT DIFFERENT COMPONENTS OF ECHOLOCATION. NO SIGNIFICANT CORRELATION WAS OBSERVED FOR ANY OF THE COMPONENTS ( $R^2$  AND  $p$  VALUES DISPLAYED ON EACH GRAPH).**

## 5.4 Discussion – Preference for gender and body condition

This study found no evidence for gender specific preference for any of the classes of echolocation calls used. If echolocation calls are used by *R. clivosus* in mate choice, one would expect listening individuals to show preference to calls of the opposite gender or from individuals with better body condition, thus facilitating sexual selection on the echolocation calls of the species. Such preferences for echolocation calls from particular individuals is a necessary requirement to infer a role of echolocation in mate choice in echolocating bats. However, despite having the ability to differentiate the calls of different genders (Finger, 2015) and body condition (Chapter 4), neither male nor female *R. clivosus* showed a preference for gender or BCI in the preference trials.

The findings of this study contrast those of Puechmaille et al. (2014) which demonstrated a potential role of echolocation in mate choice in a congeneric species. Female *R. mehelyi* apparently preferred males with high call frequencies. Puechmaille et al. (2014) argue that echolocation frequency is a reliable and honest indicator of male body condition in *R. mehelyi*, and females preferentially select males with higher frequency, concluding that male call frequency is under sexual selection. Given these findings, and assuming that *R. clivosus* follows similar methods of mate selection to the congeneric *R. mehelyi*, *R. clivosus* should show a preference for echolocation calls from individuals with relatively high BCIs. This however was not the case, instead *R. clivosus* showed no preference to either class of BCI presented in the playbacks. The use of BCI is based on the assumption that it is an indicator of fitness and therefore a point of reference for mate choice. Puechmaille et al. (2014) claimed to have validated this assumption by showing an indirect link between body condition and reproductive output using increased average colony relatedness to males with higher call

frequency (and therefore higher BCI). However, neither their study nor mine has shown directly that females mate with individuals with higher body condition. If BCI is not an indicator of reproductive output, the lack of preference by *R. clivosus* may simply be due to females not considering BCI in mate choice.

One key difference between my study and that of Puechmaille et al. (2014) is the relationship between body condition and echolocation frequency. Puechmaille et al. (2014) found a strong correlation between body condition and resting frequency in *R. mehelyi*. In contrast, *R. clivosus* shows no significant relationship between body condition and any of the parameters of echolocation measured in this study, including resting frequency. It may, therefore, be possible that the ability of *R. mehelyi* to preferentially select calls from bats with higher body condition is not universal to all horseshoe bats, but rather a consequence of the relationship between call frequency and body condition. The lack of a similar relationship in *R. clivosus* may therefore explain the lack of preference for body condition in that the bats are unable to associate a particular set of call parameters with a particular body condition.

Alternatively, the method of selection of playback calls may also have influenced the outcomes of the preference trials. In the Puechmaille et al. (2014) study, calls were selected based on their frequency, which was then assumed to represent body condition based on the correlation between body condition and call frequency. This method of selecting calls may not take into account the full range of potential acoustic cues relating to body condition. The accurate identification of high body condition may be based on more than call frequency. In my study, individual bats were individually selected based on their body condition, falling either in the top or bottom 10% of the BCI spectrum and then calls were selected from those individuals, testing the 'honest signal' assumption directly. This method ensures that all

parameters of the echolocation calls that may be used by females as indicators of BCI were included. Furthermore, Puechmaille et al. (2014) did not ensure that the frequencies of their playbacks were within the auditory range of the acoustic fovea of the female test subjects. By choosing male calls based on extremes of frequency, Puechmaille et al. (2014) may have inadvertently and artificially increased the degree of difference between the frequency of calls assigned to their high and low BCIs beyond what existed within their female test subjects. Given the reliance of horseshoe bats on the narrow range of frequency sensitivity of the acoustic fovea, this may have taken some of the playbacks outside of the hearing range of the test subjects. The apparent preferences shown by *R. mehelyi* in their study may simply reflect that some playbacks were audible (and “preferred”) while others were inaudible. Unfortunately, they do not report the call frequencies, an indirect measure of the frequencies to which the fovea is tuned, of the female test subjects. Another possible outcome of this method of selecting calls, may be that the preference observed in the study may be a result of preference for conspecifics over heterospecifics. The species used in this study, *R. mehelyi*, co-occurs with other species that use partially overlapping frequencies to their own, including the likes of *R. euryale* and *R. hipposideros*. *Rhinolophus euryale*, for instance, echolocates at an average RF of 105 kHz, whereas *R. mehelyi* echolocates 3 kHz higher, at 108 kHz (Schuchmann and Siemers, 2010b). In their study, Puechmaille et al. (2014) used calls of *R. mehelyi* split into two distinct frequency bands: a lower band (104 – 106 kHz) and a higher band (110 – 112 kHz). This lower band used coincides with the average frequency band of *R. euryale* and may therefore create some ambiguity regarding species identity. The higher band calls on the other hand fall outside the range of *R. euryale* and may therefore provide a more reliable cue for identification of a conspecific. The preference for the higher frequency calls may, therefore, be linked to a preference for conspecifics as opposed to heterospecifics.

The use of BCI as an indicator of fitness and therefore a point of reference for mate choice is based on the assumption that individuals with higher BCI sire more offspring. Puechmaille et al. (2014) showed an indirect link between body condition and reproductive output, but neither their study nor mine has shown directly that females mate with individuals with higher body condition. If BCI is not an indicator of reproductive output, the lack of preference by *R. clivosus* may simply be due to females not considering BCI in mate choice.

For echolocation to function in sexual selection, it is a prerequisite that a listening bat first be attracted to echolocation calls of the opposite gender (Ryan and Rand, 1993). In this study, neither male nor female *R. clivosus* showed any preference to the opposite gender or to their own gender, again giving equal attention to both stimuli. Interestingly, bats did show a preference for being close to conspecifics, evident by their choosing one or the other speaker from which conspecific stimuli was emitted. This preference however did not extend to any particular gender. If one is to assume that the ability of *R. clivosus* to discriminate between males and females (Finger, 2015) is indicative of recognition, then the lack of preference suggests that, in this species at least, echolocation may not be used in the selection of mates, despite being able to identify the presence of the opposite gender. An alternative explanation could be that the discriminatory ability of *R. clivosus* to echolocation calls of different genders is simply an ability to detect change, and there is no association between the calls and the gender. In this case, the lack of preference can be explained simply by bats not associating either set of calls with any particular gender, despite being able to discriminate between calls of males and females (Finger, 2015). Puechmaille et al. (2014) did not test preference between the genders. It is therefore difficult to definitively confirm if the preference for high

frequency calls is linked to mate choice. It is possible that the preference was only for higher frequency calls in general and that females would have shown equal preference for high frequency calls from other females, in which case the attribution of preference to mate choice would not necessarily stand. This, however, was not tested for by Puechmaille et al. (2014). Furthermore, if high male call frequency was the result of sexual selection one would expect that there would be at least some degree of sexual dimorphism in the call frequencies of males and females with males calling higher than females. As a matter of fact, sexual dimorphism is considered a strong indicator of potential sexual selection (Andersson, 1994; Andersson and Simmons, 2006) However, there was no sexual dimorphism in call frequency in *R. mehelyi* and in most rhinolophid species where there are gender differences, females usually call at higher frequencies than males (Neuweiler et al., 1987; Jones et al., 1992; Siemers et al., 2005; Yoshino et al., 2008; Chen et al., 2009; Jones and Siemers, 2010).

A factor which must be considered in my study is the occurrence of the mating season for *R. clivosus* in time and space. To properly investigate preference in the context of mate choice, studies need to coincide with the mating season for the species in question, as behaviours associated with preference in the context of mate choice may only be exhibited during this period (Behr and von Helversen, 2004; Voigt et al., 2008). Unlike the case of *R. mehelyi* where the exact timing of the mating season was known, the timing of the mating season for the study population of *R. clivosus* is unknown. In the case of *R. clivosus*, variation has been observed in the timing of the mating season across its southern range, with climate likely a considerable factor. In KwaZulu-Natal, a coastal region with a warm, subtropical climate, copulation is observed in May, before the onset of winter (Bernard, 1983). In Gauteng, an interior region with a mild climate and cold winters, evidence of copulation in Gauteng was observed sporadically throughout the winter months, peaking in early spring (Wessels and

van der Merwe, 1997). De Hoop Nature Reserve, in contrast, lies near the southern tip of Africa, a region exhibiting a Mediterranean climate. There has, however, been no study on the reproductive timing of *R. clivosus* in the Western Cape and the reproductive seasonality had to be estimated based on a combination of the previous studies. However, although best efforts were made to estimate the mating season, considering that the climate of the Western Cape does not match particularly well with either that of KwaZulu-Natal or Gauteng, it is possible that the reproductive timing of *R. clivosus* differs from both regions and that the study did not coincide with the exact mating season. If the study missed the true mating season, the lack of preference observed in this study may be due to test individuals not being reproductively active at the time and there may be less of a drive to preferentially select specific individuals, with bats simply investigating all acoustic stimuli equally.

In addition to the timing of the mating season, the location of reproductively active individuals may also be an influencing factor. Anecdotal evidence suggests that during the periods of this study (April/May and September), which correlate with the mating in seasons further north, reproductively active individuals are largely absent from the areas of the cave that were sampled. Only three of the 34 males captured during this time had swollen testes which were used as possible indicators of reproductive state. Furthermore, only 2 out of 36 females showed signs of previous reproductive activity (false nipples or hair-loss around the nipples), a trait commonly observed during summer months. During the summer months (November-January) *R. clivosus* can be found in large numbers throughout the cave, including reproductively mature females carrying pups (personal observation). These high numbers appear to be present until the of March, when the rainfall begins to increase and temperatures drop. After this period, however, during the spring and late autumn months when this study was conducted, the numbers appeared to be greatly reduced. The outer

chambers are devoid of almost all *R. clivosus*, with only a small number of individuals visible in the midway chambers.

At first glance, this appears to contrast the findings of McDonald et al. (1990b), who found population densities were highest during colder months. However, McDonald et al. (1990b) sampled throughout the length of the cave, including areas beyond the maternity chamber which acted as a cut-off point for our sampling (Figure 2.1). During winter months, insect abundances at De Hoop drop (McDonald et al., 1990a), limiting food availability. As a result, bats remaining at the cave enter torpor to reduce thermoregulatory costs, lowering their body temperature during periods of inactivity (McDonald et al., 1990b). To do this, rhinolophid bats roost in areas of the cave with the lowest temperatures (McDonald et al., 1990b). The region in the centre of the cave, where bats were captured in abundance during this study, may be too hot to allow bats to enter torpor, evident by the 'alert' to 'active' activity levels in the central region (McDonald et al., 1990b). *Rhinolophus clivosus* may therefore preferentially roost in the back chamber of the cave during winter months to utilize the lower temperatures to maintain energy reserves when resources are limited. The requirement for cooler body temperatures becomes more important for sexually active females, as delayed fertilization through the storage of sperm by females is thought to be an adaptation to overwintering, during periods of hibernation or reduced activity (Norris and Lopez, 2011). The presence of reproductively active females in the deep chambers of the cave may in turn attract reproductively active males into the same areas, allowing for mate choice and copulation to occur. The bats occupying the middle regions of the cave that were caught for this study may therefore be young adults who have yet to reach full sexual maturity and may therefore not engage in mate choice behaviours. The absence of the energy

requirements of reproduction, therefore, may explain why these bats are able to remain in the warmer sections of the cave without the need to enter torpor.

Given the contrasting results of this study and that of Puechmaille et al. (2014), it is still unclear exactly what role echolocation plays in preference in the context of mate choice. The system is a complex one, with multiple cues available to bats to detect and choose between potential mates, including social calls, visual, chemical and olfactory cues, all of which have been shown to function in mate choice (see (Voigt and von Helversen, 1999; Candolin, 2003; Andrews et al., 2006; Voigt et al., 2008; Bartonicka et al., 2010). These cues also need not function in isolation, with mate choice likely being based on several cues instead of just one (Candolin, 2003). There is therefore no reason to assume that echolocation must function in mate choice. The ability of bats to discriminate between sets of calls that has been attributed to a potential to recognise potential mates may simply be a result of detecting change, without recognition of context. Mate choice in bats may also require a combination of cues, meaning that although echolocation alone might not necessarily be definitive, in conjunction with additional cues it may aid in the selection of a potential mate. On the other hand, echolocation calls are a widely available resource with the potential to convey species specific cues, making it a potential cue for mate choice. Direct evidence for mate choice in bats is, however, difficult to obtain. Unlike for other acoustically mediated mating systems, such as for anurans where studies can follow the entire reproductive cycle from mate choice (which can be confirmed by observing the copulation) to gestation and test the direct reproductive outputs, studies on bats rely on indirect evidence, such as genetic analysis of relatedness to determine links between the structure of acoustic signals and reproductive success.

A potential avenue for further investigation is to investigate the role of echolocation as an isolating mechanism in mate choice. Isolating mechanisms (i.e. species-specific signals and courtship behaviours) function to prevent mating between potentially incompatible lineages to avoid potentially dysfunctional hybrids (West-Eberhard, 1983). Studies into the communicative function of echolocation, including this study, have found evidence for potential species recognition (Schuchmann and Siemers, 2010a; Li et al., 2014; Bastian and Jacobs, 2015). These studies, however, have only demonstrated an ability to discriminate between heterospecifics and conspecifics and not necessarily an ability to associate species specific signatures with a particular species which would enable them to use echolocation calls to identify conspecific individuals. Preference studies on reproductively active females presented with echolocation calls of ecologically similar heterospecifics together with conspecifics would help confirm the use of echolocation as a species specific signal.

## Chapter 6

### Synthesis and Conclusions

It is clear that horseshoe bats are adept at identifying fine details in echolocation signals and in the echoes of these calls that are reflected off objects in the environment. This is most clear in their ability to detect the faint acoustic glints in the returning echoes caused by the beating of an insect's wings (Schnitzler and Denzinger, 2011). Further demonstration of their perceptual acuity lies in their ability to discriminate between remarkably similar conspecific and heterospecific echolocation calls (Schuchmann and Siemers, 2010a; Li et al., 2014; Bastian and Jacobs, 2015). The question, however, remains if this ability to discriminate between calls extends to an ability to glean information about sender and context, or if it is simply a matter of responding to a change in stimulus.

So far, there has only been indirect support for the ACH as an explanation for signal divergence in horseshoe bats (Schuchmann and Siemers, 2010a; Li et al., 2014; Puechmaille et al., 2014; Bastian and Jacobs, 2015). Thus far it has been demonstrated that bats are able to discriminate between species based on echolocation calls, which supports the notion of echolocation functioning in communication, but there is little evidence of bats actually using this information. Puechmaille et al. (2014) demonstrated preference for echolocation calls with higher frequency in *R. mehelyi*, but only showed a link to sexual selection indirectly via a correlation between mean relatedness and peak frequency of adult males.

Over the previous three chapters I presented evidence for and against each of the three assumptions of the ACH on a single species to reach a more holistic view of the role of echolocation in communication. In this final chapter, I address all three sets of results and

discuss the impact these results have on our understanding of the role of echolocation in bat communication.

Firstly, *R. clivosus*, as with other horseshoe bats previously studied, have the ability to discriminate between species based on their echolocation calls. When signal divergence is restricted by the orientation/foraging function of echolocation, increased perceptual acuity to minor differences in acoustic signals may allow for discrete communication pathways, even between calls with overlapping frequency bands (Bastian and Jacobs, 2015). When frequencies overlapped, the ability of bats to discriminate was dependent on additional acoustic components (such as the duration, bandwidth and slope of FM Components and temporal elements of the calls) that defined the acoustic space occupied by the calls. Calls that were similar in acoustic space, such as those of *R. capensis* and the southern populations of *R. blasii*, yielded low levels of discrimination. In contrast, the calls of *R. clivosus* and more northerly populations of *R. blasii* showed greater separation in acoustic space and as a result yielded high levels of discrimination. This supports the notion that discrete communication pathways are required for successful discrimination. Presumably, considering that the distributions of *R. capensis* and *R. blasii* never overlap, their echolocation calls are not under selection for divergent acoustic space and therefore their calls are similar (Figure 3.3). As a result, *R. clivosus* is unable to discriminate between them. Alternatively, if the goal of species discrimination is to simply distinguish conspecifics from all other heterospecifics, then it would not be important for them to discriminate between two heterospecific calls, which might also explain the lack of discrimination between *R. capensis* and *R. blasii*. These findings align with the first assumption of the ACH, namely that bats can discriminate between species based on echolocation alone. However, although this demonstrates the ability to discriminate between calls, it still does not show an ability to directly associate the different calls with a

particular species. It is difficult to demonstrate a direct association on the part of the bat between a set of echolocation calls and a specific species. A potential method would be to train bats to associate calls of conspecifics and heterospecifics with food rewards on opposing sides of a two-alternative choice experimental setup. After training, the association of echolocation calls to a particular species can be tested by altering the playbacks to include calls of heterospecifics with acoustically similar calls to test if bats still associate calls of different heterospecifics with the correct arm to receive the reward.

The first experiment also highlighted the point made by Bastian and Jacobs (2015) that separation in multidimensional acoustic space is required for species discrimination. When calls occupy an overlapping frequency band, bats rely on minor changes in other components of echolocation to discriminate. This, naturally, requires an ability to detect minor changes in specific components of echolocation calls that otherwise occupy a similar acoustic space. This forms the basis of the second assumption of the ACH. Again, *R. clivosus* showed strong discriminating ability between all components investigated, including changes in call frequency, shape of FM components and duty cycle with the exception of the very similar echolocation calls of *R. blasii*<sub>87</sub> and *R. capensis*<sub>87</sub>.

With support for the first two assumptions, the basic perceptual requirements for communication appear to be in place. Bats are able to discriminate between species, BCI and gender using a number of echolocation call components. But again, this does not necessarily imply an ability to place these calls in context. For communication to shape the evolution of echolocation calls, resulting in character displacement, there would need to be an evolutionary advantage to maintaining discrete partitions in acoustic space between sympatric species. This is the basis of the third assumption of the ACH. The use of a

communication trait in mate choice is often considered an important mechanism for the evolution of the trait via sexual selection (Andersson and Iwasa, 1996; Panhuis et al., 2001; Paul, 2002).

The use of echolocation in mate choice, however, is not essential to validate the ACH. According to the third assumption of the ACH discriminating ability has to be used for reproduction or survival. Selection for discrete acoustic space and the validation of the ACH can therefore come from sexual or natural selection. Given the constraints imposed on echolocation by the functions of orientation and foraging, and the higher level of discrimination involved in sexual selection, it is more likely that selection on echolocation for communication facilitates survival, and is thus shaped by natural selection rather than sexual selection. Communication may influence the evolution of echolocation calls due to the advantages that identification of a conspecific would accrue to an individual, such as the ability to join social aggregations. Intraspecific interactions and cooperation can lead to fitness benefits for an animal (Alcock, 2001). Bats have been shown to respond to and approach echolocation calls of conspecifics in a number of situations, including during searches for food, night roosts, nursery colonies and mating sites (Barclay, 1982). The benefits of social aggregations in roost environments are known to play an important role in the evolution of bat behaviours (Kunz, 1982). The benefits of social aggregations also extend to foraging success. Energetic costs for locating rich sources of prey can be high, particularly when food sources occur in small, short lived patches separated by large areas of low density resources, as is the case for insect populations preyed upon by insectivorous bats (Gillam, 2007). Bats therefore benefit from being able to identify high quality feeding patches via eavesdropping on the echolocation calls, in particular the feeding buzzes, of other bats with

similar feeding ecology such as conspecifics (Barclay, 1982; Gillam, 2007; Ubersnickel et al., 2013).

One possible avenue to further explore the role of echolocation in communication, unfortunately not covered in this study, is preference at species level. As shown above, there are numerous evolutionary benefits to species recognition that do not involve mate choice, particularly in regards to the identification of conspecifics. To validate the ACH as an explanation for the maintenance of discrete acoustic space between species, one need only show that echolocation can be used to exercise preference for conspecifics over heterospecifics. This may already have inadvertently been demonstrated by Puechmaille et al. (2014), as argued in Chapter 5.4. Future studies should repeat preference tests to specifically include heterospecifics alongside conspecific calls to determine if preference is shown for conspecifics.

In regards to a potential role of echolocation in mate choice, further work is required to investigate exactly which traits are selected for by females and how these traits correspond with the different echolocation components. It is possible that bats simply use echolocation to detect conspecifics and switch to alternative cues (visual, olfactory, tactile etc.) when in the presence of conspecifics to actually select a mate. It is important to consider that the different components of echolocation need not act in isolation. It is likely that bats use a combination of multiple acoustic components and the interactions between these components to discriminate between and ultimately recognise species.



# Chapter 7

## References

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